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# THE EFFECTS OF GESTATIONAL EXPOSURE TO ENDOCRINE-DISRUPTING CHEMICALS ON THE ADULT SOCIAL BEHAVIOR IN MALE AND FEMALE RATS

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**by**

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# Dedication

This dissertation is dedicated to my wife, Mindy, and my parents, Mike and Pattie. Their love and support serve as the foundation for all of my achievements, now and always.

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## Abstract

# THE EFFECTS OF GESTATIONAL EXPOSURE TO ENDOCRINE-DISRUPTING CHEMICALS ON THE ADULT SOCIAL BEHAVIOR IN MALE AND FEMALE RATS

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Endocrine disrupting chemicals (EDC) exposures during critical periods of development influence neuronal development and the manifestation of sexually dimorphic behaviors that emerge in adulthood. Among these behaviors, social information processing is sexually dimorphic and regulated by sex steroids. Oxytocin and vasopressin serve as primary neurotransmitters mediating these behaviors; these neuroendocrine circuits are hormone sensitive and potential targets of prenatal EDC exposures. In dissertation, I assess the effects of gestational exposure to EDCs on the social behavior of male and females later in adulthood. A weakly estrogenic PCB mixture, Aroclor 1221, was administered to pregnant Sprague-Dawley rat dams during the time when the hypothalamus undergoes sexual differentiation. The brains of these animals were also used to quantify the presence of oxytocin or vasopressin in the two main regions of production: the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). Another experiment extended this treatment paradigm to encompass a longer period of gestational development, added another EDC treatment group

(Vinclozolin), and looked at similar behavioral outcomes. Lastly, I provide a novel way of modeling complex social behaviors in a laboratory setting. Through all of this work, we show that the sexes are differentially susceptible to endocrine disruption by PCBs or vinclozolin. Additionally, we provide evidence that the traditional choice models of social behavior in the rodent may not be reflective of how an animal behaves in a more complex, naturalistic, environment.

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# CHAPTER 1: INTRODUCTION AND BACKGROUND

## ORGANIZATION AND ACTIVATION OF THE DEVELOPING BRAIN

Throughout all of life, but particularly during the period of gestation, sex steroid hormones organize and sustain the developing brain in a sexually dimorphic manner (Phoenix et al. 1959). The functional differences between the sexes is established through specific signaling cascades which, in the case of the fetal male, are mediated by the binding of estrogen receptors (ERs) in the brain by circulating levels of testosterone (from the fetal testes) that is aromatized to estradiol in the brain. The result of this ER-mediated mechanism is a masculinization and defeminization of the developing male brain. Without developing testes, fetal females do not produce sufficient concentrations of estradiol to initiate this signaling cascade, thus the neurodevelopmental trajectory is demasculinizing and feminizing (Roselli et al. 1985). The differences in this period of sexual differentiation is known as the organizational phase of development. The differences in the development (organization) of male and female brain during the perinatal period underlie the sexually dimorphic response to gonadal hormones in adulthood (activation). The organizational-activational hypothesis presented by Phoenix et al. was perhaps one of the most crucial finding in the study of sexual differentiation. Although the activational effects of gonadal hormones were reversible, the organizational effects during a "critical period" of development were permanent.

## EXAMPLES OF SEX DIFFERENCES IN THE BRAIN AND BEHAVIOR OF MAMMALS

As determined by this critical period, the male and female developmental trajectories are forever altered. The physiological and morphological differences observed in the sexes are reflective of these changes. Not surprisingly, the area of the

brain where the highest degree of sex differences are observed are in the hypothalamic regions implicated in sexual and reproductive behaviors. One such region, the sexually dimorphic nucleus of the POA (SDN) is up to five-times larger in male rats compared to females (Gorski et al. 1980). Additional, there is a direct relationship between the size of the SDN in males and the degree of sexual behavior, with female-oriented male rams having an SDN twice the size of rams with a demaculinized preference for a male, rather than a female, partner (Roselli et al. 2004). Lesions of the SDN in rats have abolished normal partner preferences in rats (Paredes et al. 1998). The mechanism by which this sex differences arises occurs during gestation when aromatase expressed in this region in males converts circulating testosterone to estradiol, which in turn inhibits programmed cell death (Ito et al. 1986). Females, lacking sufficient concentrations of testosterone during fetal development, proceed with the apoptotic event that results in the feminized morphology and, thus behavior. Another example of a sexually dimorphic hypothalamic region directly related to prenatal gonadal steroids is the anteroventral periventricular nucleus (AVPV), which contains an impressive concentration of kisspeptin neurons that are involved in the regulation of gonadotropin releasing hormone (GnRH) neurons and the onset of the LH surge in females. In contrast to the SDN, testicular hormones in males lead to a reduction of AVPV volume in adulthood compared to females. Perinatal castration sex-reversed AVPV volume in males (Davis et al. 1996). Aside from reproductive behaviors, social behaviors are also crucial for an individual's fitness in the wild. The sexes differ in the response to olfactory cues (Bergvall et al. 1991, Aron et al. 1979). Integral to the processing of social cues, the medial amygdala (MeA) is an extra-hypothalamic brain region that is sexually dimorphic from both a structural and neurochemical perspective (Nishizuka et al. 1983, Hines et al. 1992). Driven by circulating androgens, castration decreases the volume in males, while treatment with

exogenous androgen increases the volume in females (Cooke et al. 1999). These examples illustrate the exquisite sensitivity to circulating sex steroid hormones.

## SOCIAL BEHAVIOR AND THE NEUROENDOCRINE SYSTEM

The degree to which animals socialize amongst each other runs the gamut. On one extreme, there exist solitary animals that display a high level of territorial defense against others of the same species (conspecifics). In this case, tolerance for company is reserved for two events in the animal's life: when mating, or when rearing young. On the other end of the spectrum exist highly social animals that share a living space and form communities. In mammals, high levels of complexity in the type and duration of the social interaction between each other can characterize social groups. Members of these communities must possess the ability to recognize and identify others and their emotional-state. With such complexities, there must be precise, delicate, and powerful mechanisms by which social information is processed. Specific genes and gene products that facilitate social behaviors are often sexually dimorphic, likely reflecting the differences in behavior we see amongst sexes. Among all the neurobiological systems, neuroendocrine mechanisms play a prominent role with Oxytocin (OT) and arginine vasopressin (AVP) appearing to be especially important (Donaldson et al. 2008, Lee et al. 2008, Takanagi et al. 2005). The brain distribution of OT and AVP receptors and their genes have been linked to the presence or absence of monogamy and pair bonding in voles and deer mice (Ross et al. 2009). Thus, OT and AVP appear to be key regulators of the evolution and expression of different types of social systems. OT and AVP are, in turn, under control of gonadal hormones of which the synthesis and mode of action are sexually dimorphic. Since sex steroids directly regulate gene expression, gonadal hormones mediate specific activation of different genes that can affect different social

behaviors, even when these behaviors are ultimately regulated by different neurotransmitter systems in different brain regions. (Choleris, 2008). Most research regarding the underlying neurobiological mechanisms behind social interactions is done on mice and rats in a laboratory setting. Both are polygynous (a mating system which involves one male and multiple females) species that have undergone extensive research regarding the difference in sex of their social behaviors and the underlying neurobiological mechanisms.

In order to assess the behavior of an animal in a laboratory setting, the use of standardized testing procedures is critical. By far, the most used model of social behavior in the rodent uses a three-chamber testing environment which is made up of two back-to-back scenarios that engage two separate and critical aspects of sociality (Moy et al. 2004). In each scenario, a binary choice of immobile stimulus options allows an investigatory to observe how a mobile subject animal spends it's time within a social context. Typically, the first test pairs a non-social stimulus with a social stimulus to gain insight into the subject's affiliative nature. In the case of rats, the expected phenotype is to spend more time near/interacting with the social stimulus compared to the empty cage. During this test, the subject acquires a sense of familiarity (habituation) to the conspecific used as the social stimulus. The next stage of the test takes advantage of this by introducing a novel stimulus animal. The expected phenotype in rats during this phase of testing is for the subject to spend more time affiliating with the novel conspecific over the familiar. While the first stage of testing only provides insight into the general affiliative nature of a subject, the second test also engages the subject's social memory as well as discriminatory abilities. The ubiquitous nature of this paradigm and comparisons across studies allow investigators to assess the social implications of a variety of experimental

manipulations. Such experimental manipulation has implicated the importance of the OT, AVP, and gonadal steroids on social recognition and discrimination.

**The Influence of Estrogens On Social Discrimination**

Gonadal hormones have been shown to be involved in social recognition, with modulation occurring through OT and AVP systems. Generally, in female mice, social recognition is increased during proestrus, when estrogen (E) and progesterone (P) levels are high. Intuitively, this makes sense as increased social recognition would be beneficial or necessary when reproductively active. Ovariectomy (OVX) leads to decreased social recognition in both rats and mice, but treatment with E can recover the deficit (Tang, 2005). Additionally, ERαKO and OTKO females are completely impaired, while ERβKO mice maintain some amount of discrimination (Choleris, 2006). Thus, ERα and OT appear to be necessary for social recognition, while ERβ only facilitates it. Since levels of OT and OT mRNA levels depend on the presence of E, it has been proposed that E controls social recognition through the OT system. OT and OT mRNA levels fluctuate with the estrous cycle in a manner consistent with the fluctuating levels of E. Similarly, OVX animals have reduced levels of OT and OT mRNA, however this is not a complete reduction. Administration of estrogens directly regulated OT production by increasing the excitability of OT-producing neurons in the PVN. This is probably mediated by ERβ since its expression in the PVN is much higher than ERα. More evidence for this exists in the fact that ERβKO male and females do not show increased levels of OT in the PVN when estrogens are administered. Baseline levels of OT and OT mRNA for the OT gene in the PVN are otherwise normal. The baseline OT may be involved in the decreased disruption of social recognition in ERβKO animals. Treatment with estrogens also increased OTR density (as well as transcription of the gene encoding for OTR) in the

medial amygdala; this is where OT mediates social recognition. ERα is highly expressed in the medial amygdala and is required for the induction of the OTR. ERβ is also highly expressed in this brain region, but it is not required for transcription of the OTR gene. Also, binding of OT to the OTR is independent of ERβ. This could explain why ERβKO animals are still able to perform in the social discrimination paradigm. RNA interference of the OTR gene in the medial posteriodorsal amygdala blocks social recognition.

The basis of estrogenic control of OT-mediated social recognition has been placed on four genes in two brain areas; known as the micronet model (Choleris, 2003). The four genes involved in the model encode for ERα, ERβ, OT, and OTR. The two brain regions involved are the PVN and medial amygdala. There must be delicate interplay between these genes and regions to result in normal social recognition. Estrogens control OT production in the PVN through ERβ while also controlling the expression of the OTR gene in the medial amygdala, where socially relevant olfactory information is processed. Pharmacological studies looking at rapid activation of ERα and ERβ by using their respective agonists prior to behavioral testing showed increased social recognition in only animals treated with PPT (ERα agonist) and not DPN (ERβ agonist) (Phan, 2011). This suggests that ERβ does not affect social recognition when acting through rapid, non-genomic means. It also suggests that PPT activated (MAPK)-dependent signaling cascades, which are involved in synaptic plasticity and the formation of new memories (Thomas, 2004). The rapid effects of PPT are also seen in other forms of learning, so this is not specific to social recognition.

**The Influence of Androgens on Social Discrimination**

Androgens are also suggested to play a role in social recognition. When exposed to a previously encountered juvenile animal, intact males only exhibit habituation when the second exposure is less than 60 minutes after the first. However, females and castrated males still exhibit habituation when the second exposure was three hours later. This suggests that intact males are less able to recognize familiar conspecifics. There exists a transient decreased in social recognition in castrated males post-castration that is fully recovered if the animals are tested 2-3 weeks post-surgery. Overall, androgens are indicated in being involved in social recognition, however the effects of castration depend on the timing of the testing as well as how many times the animals were tested post castration.

Prenatal treatment with the androgen receptor (AR) antagonist flutamide did not affect social recognition in male rats. Thus, if testosterone (T) regulates social recognition in male rats, it does not do so via AR-mediated developmental effects. Studies in mice show an involvement of T on social recognition through estrogenic mechanisms (Pierman, 2008). Aromatase knockout (ArKO) male mice, with a mutated cyp19 gene resulting in an inability to aromatize T to E, have impaired social recognition. This suggests that the impairment of social recognition in the ArKO mice was due to activational and not organizational effects of the gene KO. ArKO males with impaired social recognition also had high levels of T, which could lead to two different interpretations. One possibility is, simply, E is important for social recognition in males. The other being that T's activation of the AR impairs social recognition. More extensive studies regarding the role of T and AR in social recognition are required to further clarify this relationship.

With AVP expression being much higher in males, androgen effects on social recognition is likely mediated by AVP and regulated by circulating T. Castration reduces AVP in several limbic brain areas in a manner that can be reversed by treatment with testosterone. Brain areas where AVP neurons are T-dependent are the BNST and the medial amygdala, which both project to the LS where AVP action is required for social recognition in males. Thus, AVP-enhancing effects of T may be mediated by its aromatization to estrogens. ArKO males had reduced AVP in the medial amygdala, LS, BNST, and the SON (Pierman, 2008).

Studies with ERαKO and AR-mutated mice suggest that both ERα and AR contribute to AVP expression in the limbic system (Scordalakes, 2004). In mice, estrogens induce a reduction of AVP in the PVN, which is opposite to the estrogen-induced release of OT in the PVN. This effect was suppressed in ERβKO mice, suggesting a dual and opposite role of ERβ on OT and AVP synthesis in the PVN. Females and castrated males (who have a reduction in AVP projections from both the BNST and medial amygdala to the LS) still display social recognition that is not dependent on the AVP as evident by no change when subjected to an agonist or antagonist for the AVP receptors. AVP dependence of social recognition can be restored only in castrated males by treatment with T. Thus, it seems that, while treatment with T and E-dependent AVP mediates social recognition in male rats and mice, AVP is not essential for social recognition and alternate pathways can mediate the behavior when this pathway is disrupted.

Complex involvement of OT, AVP their receptors, as well as sex steroid modulation serve as the bases for the complexities we can observe in social behavior in animals. With sex differences in AVP expression and sex hormone production, we can begin to infer mechanisms regarding the different emergent behaviors seen amongst

males and females. With a further understanding of the intricacies involved in the relationships between the brain, hormones, and behavior, it will be possible to develop more nuanced behavioral paradigms that aim toward a more naturalistic approach. As the mechanisms underlying social behavior become clearer, it will allow development of potential therapies aimed at improving deficits seen in various aspects of social behavior. In fact, Larry Young's lab at Emory University has been interested in developing a model for the autism spectrum disorder (ASD) by employing a genetically modified prairie vole. While pharmacological treatments for ASD do not yet exist on the market, this system appears a likely prospect as an avenue by which therapies for disruptions in social behaviors may be addressed.

## ENDOCRINE-DISRUPTING CHEMICALS

For better or worse, Earth is subject to the innovations of it's inhabitants. Through decades of agricultural, industrial, and technological advancements, humans have made a mark on this planet at the cost of environmental contamination. One such class of chemical contaminants are endocrine-disrupting chemicals (EDCs), which are defined as an "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Gore et al. 2014). Particular to the chemical properties of any one EDC, biological action is mediated through a variety of mechanisms which may produce adversities in developmental, reproductive, cardiovascular, metabolic, and immune function in humans (Schug et al. 2011). Implicated in the mechanism of action of many EDCs are the wide-spread thyroid, androgen, or estrogen -sensitive tissues, interference of which can lead to functional changes in receptor signaling and/or circulating concentrations of steroid hormones (Dickerson and Gore 2007; Bellingham et al. 2012). EDCs also have been shown to exert effects not directly mediated via steroid

receptor systems, but also through interference with neurotransmitter systems, steroid metabolism, or steroidogenic enzyme function (Diamanti-Kandarakis et al. 2010).

**Polychlorinated Biphenyls (PCBs) and Vinclozolin (VIN)**

PCBs are a ubiquitous and mixture of EDCs that have contaminated the environment after decades of industrial use as a lubricating and insulating compound. Although PCB usage was banned in America in 1979, these organochlorides are known to persist in the environment and still pose equal or, due to bioamplification, greater risks to the environment and its inhabitants. Exposure via ingestion or inhalation has led to detectible levels of PCBs found in humans, particularly in those living in densely populated urban areas (Sun et al. 2007). Through human epidemiological studies, there have been linkages between exposure to mixtures of PCBs and various health adversities, including decreased performance on standardized testing in children born of mothers around the Great Lakes region, where there is an increased incidence of PCB-contaminated fish consumption (Stewart et al 2008). The specific actions of PCBs depend highly on the degree of chlorination, which has an influence on the overall structure (and function) of the molecule (Giesy and Kannan 1998). One class, dioxin-like PCBs, exert their toxic effects primarily through the aryl hydrocarbon receptor (AhR) system (Zhang et al. 2012). Non-dioxin-like PCBs do are not processed by the AhR but rather induce their effects on the neuroendocrine systems, and it is these congener mixtures which have been implicated in interference the neuroendocrine system via estrogenic mechanisms (Arcaro et al. 1999, Shekhar et al. 1997). Aroclor 1221 (A1221) is the trade name of one such non-dioxin-like PCB mixture which is known to have estrogenic properties. This dissertation will focus on A1121 which has been shown to interfere with brain mechanisms that modulate anxiety, social, and reproductive behavior in adulthood

(Steinberg et al. 2007, Dickerson et al. 2011, Walker et al 2012, Topper at al. 2015, Reilly et al. 2015, Gillette et al 2017).

Vinclozolin (VIN) is another compound classified as an EDC. This Fungicide is still widely used on food crops destined for human consumption (Cabras and Angoni 2000). Through experimentation in the laboratory, VIN has been classified as an anti-androgenic EDC. In rodent studies, exposure to VIN has been shown to result in alterations of mechanisms and behaviors mediated by the androgen receptor resulting in morphological abnormalities in both male and female rodents exposed during gestation (Ostby et al. 1999, Crews et al. 2000, Wolf et al. 2000, Buckley et al. 2006).

## EDCs and Social Behavior

There is a growing body of literature that correlate developmental perturbations via EDCs on the acquisition and manifestation of appropriate social behaviors. In particular, exposure to bisphenol A (BPA), phthalates, and PCBs have resulted in measurable differences in social behaviors in rodents. Pre or Perinatal exposures to BPA resulted in reduced territorial marking in male mice (Williams et al. 2013), altered novelty preference (Wolstenholme et al. 2013), reduced play and social grooming in females (Porrini et al. 2005), but increased play in males (Farabollini et al. 2002). Rats exposed to phthalates displayed abnormal social behaviors (BPP; Betz et al. 2013) and a reduction in copulatory behavior in both sexes (DBP, DINP, DEHA; Lee et al. 2006). Rats exposed to PCBs (congener 77) showed a decreased preference for maternal-associated cues, but no impact on novel (odor) preference(Cromwell et al. 2007). Rats given a mixture of PCBs (congeners 47 and 77) prenatally showed impairments in social recognition (Jolous-Jamshidi et al. 2010). For the most part, many of the social

behavioral outcomes associated with BPA, Pthalate, or PCBs are sex-specific, suggesting that the sexes may be differentially susceptible.

In general, the processes that govern the sex differences in the brain and behavior of mammals is sensitive to the actions of hormones during development. This critical period is particularly sensitive to the actions of EDCs and there is a growing body of literature that have shown how EDC interference may lead to life-long alterations in adult social behavior.

## SUMMARY OF DISSERTATION EXPERIMENTS AND OVERARCHING HYPOTHESIS

This dissertation aimed to develop an in-depth behavioral characterization of animals exposed to EDCs during these critical periods of sexual differentiation during embryonic development. Observing both sexes allowed me to determine where the sexes differ in their behavior and test the overarching hypothesis that these sex differences present dissimilar susceptibilities to endocrine disruption.

**Chapter 2 tested the hypothesis that prenatal exposure to PCBs lead to observable changes in the adult social behavior in male and female rats.**

Here, I presented pregnant rats with one of two low-dose (biologically relevant) intraperitoneal injections of PCBs during the time when the hypothalamus was undergoing sexual differentiation. Following parturition, offspring were monitored for somatic and sexual development. After puberty, the animals were tested in the three-chamber social test to see if PCB exposure led to any changes in affiliative or discriminatory behavior by using same-sex gonadectomized conspecific stimulus animals.

**Chapter 3 examined whether other EDCs and a longer duration of exposure affect social behaviors.**

In addition to the inclusion of another EDC (Vinclozolin) this chapter allowed to me see if a longer duration of EDC exposure led to outcomes that differed from the dosage paradigm presented in chapter 2. Although the social behavioral testing paradigm used here was the same as chapter 2, this chapter used same-sex intact stimulus animals.

**Chapter 4 presented a novel social environment in order to determine if the complex social setting provides further insight into the behavioral differences due to PCB exposure**

This chapter used the same dosage paradigm as chapter 2, but here I present a novel behavioral paradigm that doubles the number of stimulus choices available to the experimental animal. This allowed me to determine the influence of both sex and hormone on the affiliative properties of an animal exposed to PCBs during gestation.

**Chapter 5 tested whether changes in behavior due to prenatal PCBs resulted in changes in the brain.**

Using the brains of the animals that were behaviorally characterized in chapter 2, this chapter looks at the paraventricular and supraoptic nucleus (PVN and SON) of both sexes to see if any alterations in behavior can be related to the amount of oxytocin or vasopressin in the main sites of synthesis.

# CHAPTER 2: THE EFFECTS OF PRENATAL PCBS ON ADULT SOCIAL BEHAVIOR IN RATS

The text in this section is excerpted from Reilly MP, Weeks CD, Topper VY, Thompson LM, Crews D, Gore AC, Hormones and Behavior (2015), with permission from the journal. As first author, I was involved with all of the experimentation, analysis, and preparation of the manuscript.


## ABSTRACT

Endocrine disrupting chemical (EDC) exposures during critical periods of development may influence neuronal development and the manifestation of sexually dimorphic sociability and social novelty behaviors in adulthood. In this study, we assessed the effects of gestational exposure to PCBs on the social behavior of males and females later in adulthood. A weakly estrogenic PCB mixture, Aroclor 1221 (A1221, 0.5 or 1 mg/kg) was administered to pregnant Sprague-Dawley rat dams. Both a positive control (estradiol benzoate; EB, 50 μg/kg) and negative control (dimethylsulfoxide; DMSO in sesame oil vehicle) were similarly administered to separate sets of dams. The sexes responded differently in two tasks essential to sociality. Using a three-chamber apparatus that contained a caged, same-sex, gonadectomized stimulus animal and an empty stimulus cage, we found that both sexes showed a strong preference for affiliating with a stimulus animal (vs. an empty cage), an effect that was much more pronounced in the males. In the second task, a novel and a familiar stimulus animal were caged at opposite ends of the same apparatus. Females displayed a higher degree of novelty preference than the males. During both tests, females had significantly higher social approach behaviors while male engaged in significantly more interactive behaviors with the conspecific. Of particular interest, males born of dams that received prenatal A1221 (0.5 mg/kg) exhibited an overall decrease in nose-to-nose investigations. These behavioral data suggest that the males are more sensitive to A1221 treatment than are

females. In addition to behavioral analysis, serum corticosterone was measured. Females born of dams treated with A1221 (0.5 mg/kg) had significantly higher concentrations of corticosterone than the DMSO female group; males were unaffected. Females also had significantly higher corticosterone concentrations than did males. Overall, our results suggest that the effects of gestational exposure to PCBs on adult social behavior are relatively limited within this particular paradigm.

## INTRODUCTION

Prenatal exposure to endocrine disrupting chemicals (EDCs) can disrupt the neuroendocrine system, leading to alterations in adult social and sociosexual behaviors in a sexually-dimorphic manner. Most research has been conducted for bisphenol A (BPA), exposure to which causes a decrease in the territorial marking of male mice (Williams et al. 2013), as well as female-specific alteration of one-on-one social interactions in juvenile mice (Wolstenholme, 2011) and prairie voles (Sullivan et al. 2014). BPA also perturbs social recognition in mice (Wolstenholme, Goldsby, and Rissman 2013). Exposure to other EDCs such as atrazine (mice: Belloni et al., 2011), PCBs (rats: Jolous-Jamshidi et al., 2010), and chlorpyrifos (mice: Venerosi et al., 2012) are associated with perturbations of normal social interactions. Polychlorinated biphenyls (PCBs) - including the Aroclor 1221 mixture (A1221) used in the current study – also disrupt sexual behavior in female rats (Chung and Clemens, 1999; Steinberg et al., 2007). However, beyond this work, studies of EDC effects on social affiliation (individual preference to associate with a conspecific) and social novelty (individual choice to affiliate with a strange versus a familiar conspecific) are limited. Research has shown sex differences in these behaviors, as male rats tend to spend more time interacting with an unfamiliar,

same-sex conspecific than do females (Carrier and Kabbaj 2012; Slamberová et al. 2011; Stack et al. 2010). However, to our knowledge there are no studies investigating the effects of gestational exposure to PCBs on this paradigm.

The purpose of this study was to provide a thorough characterization of the social behavioral phenotype caused by gestational EDC exposure. We assessed how treatment of a pregnant rat dam with A1221 during the third trimester of gestation affected the social behavior of male and female offspring later in adulthood. Two dosages of A1221 (0.5 and 1 mg/kg) were administered during the last trimester of gestation, during a critical period of sexual differentiation of the hypothalamus (Davis, Popper, and Gorski 1996; Jacobson et al. 1980). Both positive control (estradiol benzoate; EB) and negative control (DMSO vehicle) groups were used for comparison. Using this model, we were able to address hypotheses about sex differences in performance in two types of socially relevant tests, and to test the hypothesis that prenatal exposure to EDCs in these responses would have sex-specific effects. Because it is known that male and female rats differ in their basal concentrations of corticosterone (Kitay, 1961; Gillette et al., 2014) and, further, that circulating levels of corticosterone influences social behaviors in rats (Veenit et al. 2013), we also measured concentrations of this hormone in our experimental rats.

## MATERIALS AND METHODS

### Experimental Design.

Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Houston, TX), and all animal procedures were conducted in compliance with protocols approved

by IACUC at the University of Texas at Austin. They were housed in a colony room with controlled temperature (22 C) and light cycle (12:12 dark:light, lights on at 2400). Virgin females were mated with sexually experienced males. The day following successful mating, as indicated by a sperm-positive vaginal smear, was termed embryonic day 1 (E1). Male and female stimulus rats were purchased as young adults from Harlan, and gonadectomized under isoflurane anesthesia. Stimulus animals were not treated with EDCs or vehicle.

Pregnant rats were exposed to one of four treatments, administered via intraperitoneal injections, on E16 and E18, the beginning of the period of brain sexual differentiation(Davis, Popper, and Gorski 1996; Jacobson et al. 1980). The dosages used were based on prior work conducted in the Gore lab that showed physiological, behavioral, and neuroendocrine effects (Steinberg et al., 2007, 2008; Dickerson et al., 2011a; Walker et al., 2013, 2014): (1) Vehicle (3% DMSO/sesame oil mix), (2) Estradiol benzoate (EB; 50 μg/kg), (3) Aroclor 1221 (A1221, 0.5 mg/kg), or (4) A1221 (1 mg/kg). The number of litters per treatment was 11, 11, 10, and 10, respectively. Although we did not measure body burden or tissue content in the exposed offspring, the literature suggests that maternal-fetal transfer results in an exposure to approximately 1-2 μg/kg A1221, and 100 ng/kg EB, in the fetuses (Takagi et al., 1986).

The day of parturition was called postnatal day 0 (P0). At P1, the newborn pups were weighed and their anogenital distance measured; litters were culled to 4 males and 4 females. The pups were monitored daily for eye opening, while body weights and anogenital distance were taken weekly following birth. The pups were weaned at P21 and rehoused in same-sex groups where they were monitored daily for signs of pubertal

development: vaginal opening in females and preputial separation in males (Steinberg, Juenger, and Gore 2007; Walker et al. 2012). Following vaginal opening, daily vaginal smears were taken and cell cytology was examined as a measure of estrous cyclicity in the females. Beginning at P60 animals were subjected to a battery of the following tests in random order: sociability and social novelty, mate preference, open field and elevated plus maze; fear conditioning always was the last test. The total number of behaviorally characterized animals was 82 females and 80 males. Order of testing had no effect on behavioral outcomes. Experimental rats were weighed and euthanized 30 days after testing was completed, and bloods centrifuged and frozen for hormone assay, and adrenals and gonads removed and weighed.

**Hormone Radioimmunoassay**

Around P90, animals were euthanized by rapid decapitation and trunk blood was collected; females were euthanized in proestrus. In addition, animals from the same litters that were not behaviorally tested were used to increase sample size; this resulted in a total number of 158 females and 153 males. 10 μl of sample from each individual was used to measure serum corticosterone concentration in a single non-human radioimmunoassay (MP Biomedicals; Corticosterone 3H RIA - 07120002). Assay sensitivity was 7.7 ng/ml, and intra-assay variability was 4.1%.

**Behavioral Paradigm**

A three-chamber social apparatus (100 cm x 100 cm; Stoelting, Figure 2.1) was used as the testing arena (Crews et al. 2012; Moy et al. 2004). Testing was conducted under dim red light during the dark period of their light-dark cycle, approximately two

hours following lights out. The experimental animal was placed in the middle chamber of the apparatus, with doors to the two side chambers closed. For females, estrous cycle status on the day of testing was recorded to identify any potential differences relating to the behaviors examined. Same-sex gonadectomized stimulus animals were placed in a 7 cm x 15 cm cylindrical stimulus cage located in a corner of the lateral chambers; bars allowed for nose-to-nose investigation but did not permit further contact.

**Sociability and Social Novelty.**

A five minute habituation period was used to allow the experimental rats access to the center chamber only. The doors were then opened and the experimental animal allowed to freely move around the entire apparatus for the two ten minute periods. All behaviors were video recorded throughout the testing. The entire apparatus was dismantled and all surfaces wiped clean with a 70% ethanol solution between each test. During the first Sociability test, one of the stimulus cages, randomly selected, held a novel same-sex (untreated by EDCs, and gonadectomized in adulthood) rat while the other stimulus cage remained empty (Figure 2.1A). At the test's conclusion, the experimental animal was removed from the apparatus and temporarily placed in a holding cage. The original stimulus rat, and a novel same-sex, gonadectomized stimulus animal, were each placed into stimulus cages and were randomly placed into opposite sides of the testing arena. The experimental animal was then reintroduced to the center chamber, marking the beginning of Social Novelty. The experimental animal was then allowed to interact with the now-familiar and novel stimulus animals for ten minutes (Figure 2.1B).

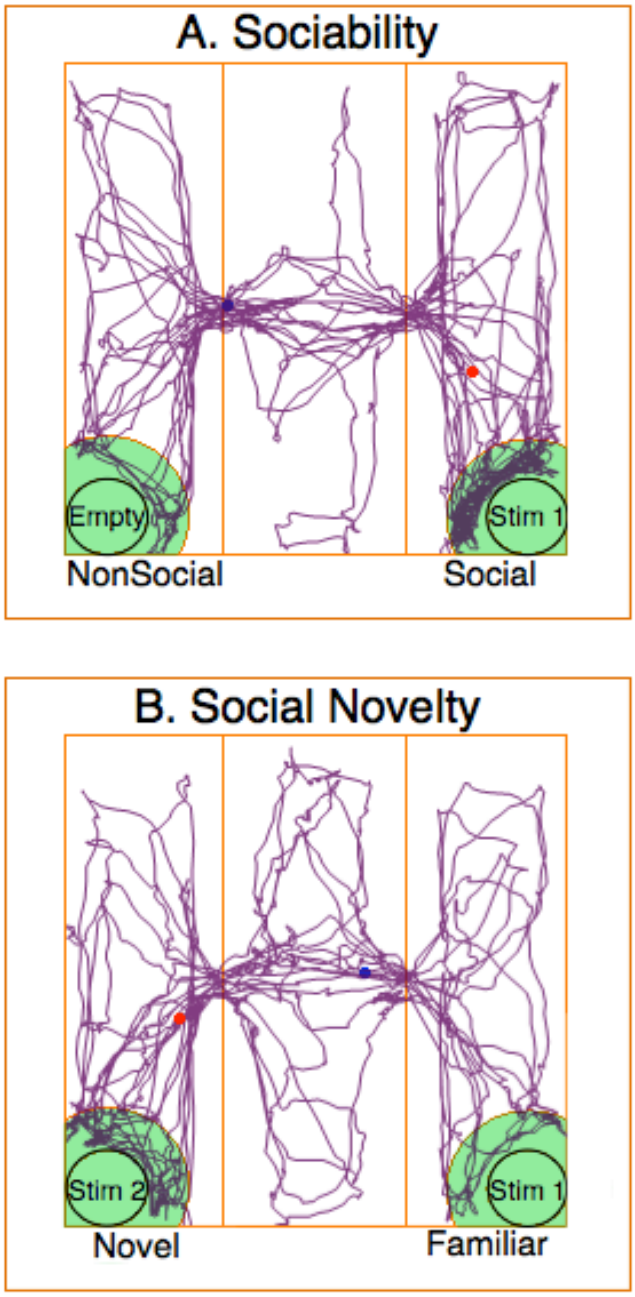



Figure 2.1: The Three-Chamber Apparatus

A diagram of the 3-chamber apparatus, with a representative tracking profile from Any-Maze for an individual rat, is shown for the Sociability (A) and Social Novelty (B) tests. In Sociability (A), the stimulus rat (Stim 1) was a same-sex, gonadectomized rat, and the other cage was empty. In Social Novelty (B), the same animal (Stim 1) was used again as the familiar rat, together with an unfamiliar same-sex, gonadectomized rat in the other cage (Stim 2).

AnyMaze (Stoelting Co.) was used to track behaviors. Automated computer-scored measures were: total distance travelled, and average speed throughout the entire apparatus. The time in proximity (defined as one body length) to the stimulus cage was also determined by the program. The video recordings of the tests were manually scored for the following behaviors: nose touching (the time each experimental animal spent in direct nose-to-nose contact with the stimulus animals), stimulus rat investigation (the time spent investigating the stimulus animal, but not necessarily nose-touching), grooming (time spent self-grooming), and rearing (time spent on hind legs without support from any walls).

**Statistical Analyses**

Because of non-homogeneity of behavioral datasets, the Kruskal-Wallis test was used to compare effects of treatment within sexes. A generalized extreme studentized deviate (ESD) test was used to detect outliers, limited to a maximum of two per group. Any animals that were outstanding outliers across multiple endpoints were removed from the analyses. Posthoc analyses included t-test for sex effects, Tukey HSD for treatment effects within sexes, or Steel-Dwass for treatment effects within sexes when the data did not satisfy the assumptions for parametric analyses. Cohen's d analysis was used to determine the effect size, within each group, for the Social Novelty data. An effect size of 0.8 or higher is equivalent to Cohen's standard LARGE, and indicates that the mean of the control group (Familiar) is at the 79th percentile and sharing 69% overlap with the comparison group (Novel). The hormone data were homogeneous and a two-way ANOVA identified main effects of treatment and sex; subsequent one-way ANOVA was performed to determine the effects of treatment within sexes. Initial statistical analyses

were used to identify any potential cohort or litter effects within groups; none were identified, and therefore, analysis was conducted using individuals within a litter as separate datapoints (no more than 2 per sex per litter). This resulted in 10-11 litters per treatment, with 20-22 males and 20-22 females per endpoint for behaviors.

## RESULTS

There were no significant effects of female estrous cycle status, corticosterone concentration, litter, or cohort on any of the behavioral measures examined for Sociability or Social Novelty.

### Corticosterone

A two-way ANOVA indicated a sex difference ($F_{1, 312} = 114.01$, $p < 0.0001$), with females having a significantly higher serum concentration than males (Figure 2.2). Subsequent analyses of effects of treatment within each sex showed significant differences in females ($F_{3,154} = 4.13$, $p < 0.009$); a Tukey HSD post hoc test found the A1221 (0.5 mg/kg) group to have significantly higher corticosterone concentrations than the DMSO group. There were no significant differences among the male treatment groups.

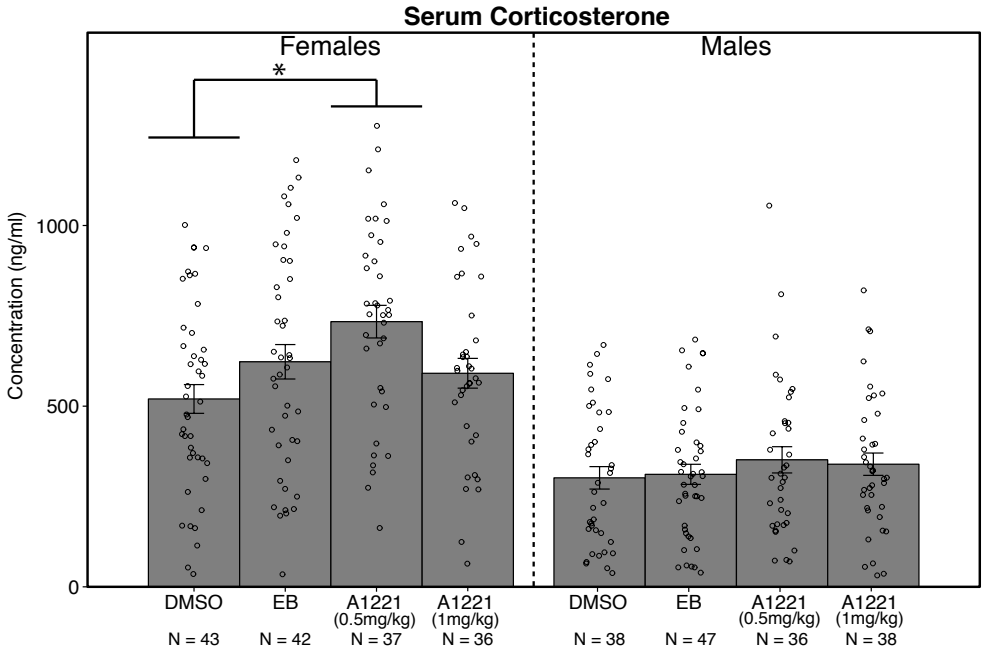



Figure 2.2: Serum Corticosterone Concentration For Both Sexes

Circulating concentrations of corticosterone are shown for adult males and females receiving prenatal exposure to the vehicle (DMSO), estradiol benzoate (EB) or Aroclor 1221 (A1221, 0.5 or 1 mg/kg). Females had significantly higher corticosterone concentrations than males ($p < 0.0001$). Within females, A1221 (0.5 mg/kg) rats had significantly higher concentrations of serum corticosterone than the DMSO females. Data shown are mean $\pm$ standard error, with individual values shown as circles. *, $p < 0.05$. N's are indicated below each bar.

**Body weight**

A Student's t-test indicated that males ($\bar{x}$ = 415 g) weighed significantly more than females ($\bar{x}$ = 264 g), regardless of treatment ($p < 0.0001$). A one-way ANOVA within each sex revealed that rats that were prenatally treated with either dosage of A1221 had significantly greater body weight ($p < 0.001$) at the age at euthanasia (~P90; Table 2.1).

**Adrenal gland weight**

A two-way ANOVA (Sex x Treatment) indicated a main effect of sex, with females ($\bar{x}$ = 0.06 g) having heavier adrenals than males ($\bar{x}$ = 0.05 g), regardless of treatment ($p < 0.0001$; Table 2.1). However, there were no significant effects of treatment on adrenal weight in either sex.

**Gonad weight**

One-way ANOVA within each sex indicated no effect of treatment on ovarian weight in females, or testicular weight in males (Table 2.1).

| Measure | Sex | Treatment | Mean | SEM | Sex Diff. |
|---|---|---|---|---|---|
| Body weight (g) | Females | DMSO | 257 | ± 4 | F < M p < 0.0001 |
| | | EB | 263 | ± 3 | |
| | | A1221 (0.5) | 270 | ± 2 | |
| | | A1221 (1) | 265 | ± 3 | |
| | Males | DMSO | 400 | ± 9 | |
| | | EB | 413 | ± 6 | |
| | | A1221 (0.5) | 427 | ± 7 | |
| | | A1221 (1) | 419 | ± 4 | |
| Adrenal gland weight (g) | Females | DMSO | 0.057 | $\pm 1.7 \times 10^{-3}$ | F > M p < 0.0001 |
| | | EB | 0.059 | $\pm 1.8 \times 10^{-3}$ | |
| | | A1221 (0.5) | 0.065 | $\pm 2.5 \times 10^{-3}$ | |
| | | A1221 (1) | 0.062 | $\pm 1.5 \times 10^{-3}$ | |
| | Males | DMSO | 0.048 | $\pm 1.4 \times 10^{-3}$ | |
| | | EB | 0.05 | $\pm 1.1 \times 10^{-3}$ | |
| | | A1221 (0.5) | 0.049 | $\pm 1.2 \times 10^{-3}$ | |
| | | A1221 (1) | 0.052 | $\pm 2.1 \times 10^{-3}$ | |
| Gonad weight (g) | Females | DMSO | 0.13 | $\pm 4.7 \times 10^{-3}$ | N/A |
| | | EB | 0.13 | $\pm 4.0 \times 10^{-3}$ | |
| | | A1221 (0.5) | 0.13 | $\pm 3.7 \times 10^{-3}$ | |
| | | A1221 (1) | 0.13 | $\pm 5.0 \times 10^{-3}$ | |
| | Males | DMSO | 4.0 | $\pm 5.0 \times 10^{-2}$ | N/A |
| | | EB | 4.2 | $\pm 6.5 \times 10^{-2}$ | |
| | | A1221 (0.5) | 4.0 | $\pm 5.7 \times 10^{-2}$ | |
| | | A1221 (1) | 4.1 | $\pm 5.0 \times 10^{-2}$ | |

Table 2.1: Somatic Measures in Prenatally Exposed Rats

Body weight, adrenal weight, and gonad weight were sexually dimorphic. Prenatal treatment with vehicle (DMSO), estradiol benzoate (EB), and Aroclor 1221 at 0.5 or 1 mg/kg did not affect these endpoints. Data shown are mean + SEM.

**Sociability Test**

Sociability tests were completed on 82 females and 80 males. Three animals (all male) that never entered the chamber containing the stimulus animal during the first stage (Sociability) had to be excluded, as they had never interacted with the animal and could therefore not distinguish a novel from a familiar conspecific. The computer-generated data for the diagnostic behaviors of the Sociability tests are shown in Table 2.2A, and investigator-scored data related to behaviors that took place in proximity to a stimulus animal are shown in Table 2.3A. Main effects of sex were determined by grouping the treatment groups within each sex and running a Student's t-test, with results shown in Tables 2.2A and 2.3A. Females traveled a greater distance, were faster, and engaged in more grooming and rearing than males. Males had longer latencies to investigate the stimulus animal and to engage in the first nose touch, than did females. Males also spent more time investigating the stimulus animal and nose touching than females. The sexes were equivalent in time spent in proximity to the stimulus rat. Although rats spent more time in the chamber containing a stimulus animal than the empty chamber, regardless of sex or treatment ($H = 197.40$; $p < 0.0001$), within each sex there were no treatment effects. Two representative behaviors are shown in Figure 2.3 for latency to investigate the stimulus animal, and time spent nose touching, illustrating the sex difference but no significant treatment effects.

A. Sociability

| Measure | Sex | Treatment | Mean | SEM | Sex Diff. |
|---|---|---|---|---|---|
| Distance (m) | Females | DMSO | 53.0 | ± 2.6 | F > M p < 0.0001 |
| | | EB | 53.0 | ± 2.2 | |
| | | A 0.5 | 52.0 | ± 2.6 | |
| | | A 1.0 | 57.4 | ± 4.7 | |
| | Males | DMSO | 37.7 | ± 2.8 | |
| | | EB | 48.4 | ± 4.9 | |
| | | A 0.5 | 46.0 | ± 2.4 | |
| | | A 1.0 | 39.0 | ± 2.4 | |
| Speed (cm/s) | Females | DMSO | 8.8 | ± 0.4 | F > M p < 0.0001 |
| | | EB | 8.9 | ± 0.3 | |
| | | A 0.5 | 8.7 | ± 0.4 | |
| | | A 1.0 | 9.6 | ± 0.8 | |
| | Males | DMSO | 6.3 | ± 0.4 | |
| | | EB | 8.1 | ± 0.8 | |
| | | A 0.5 | 7.7 | ± 0.4 | |
| | | A 1.0 | 6.3 | ± 0.4 | |
| Grooming (s) | Females | DMSO | 6.6 | ± 1.2 | F > M p < 0.05 |
| | | EB | 8.1 | ± 1.9 | |
| | | A 0.5 | 6.3 | ± 1.3 | |
| | | A 1.0 | 4.1 | ± 0.7 | |
| | Males | DMSO | 3.6 | ± 1.1 | |
| | | EB | 4.7 | ± 1.0 | |
| | | A 0.5 | 3.3 | ± 0.9 | |
| | | A 1.0 | 5.7 | ± 1.5 | |
| Rearing (s) | Females | DMSO | 6.2 | ± 1.3 | F > M p < 0.03 |
| | | EB | 4.2 | ± 1.2 | |
| | | A 0.5 | 5.5 | ± 1.5 | |
| | | A 1.0 | 4.2 | ± 1.0 | |
| | Males | DMSO | 2.8 | ± 0.9 | |
| | | EB | 3.3 | ± 0.9 | |
| | | A 0.5 | 1.9 | ± 0.4 | |
| | | A 1.0 | 2.3 | ± 1.6 | |

B. Social Novelty

| Measure | Sex | Treatment | Mean | SEM | Sex Diff. |
|---|---|---|---|---|---|
| Distance (m) | Females | DMSO | 46.6 | ± 2.3 | F > M p < 0.0001 |
| | | EB | 48.4 | ± 3.1 | |
| | | A 0.5 | 45.5 | ± 2.4 | |
| | | A 1.0 | 48.6 | ± 2.6 | |
| | Males | DMSO | 33.9 | ± 2.1 | |
| | | EB | 33.7 | ± 2.2 | |
| | | A 0.5 | 41.0 | ± 3.0 | |
| | | A 1.0 | 36.6 | ± 2.2 | |
| Speed (cm/s) | Females | DMSO | 7.9 | ± 0.4 | F > M p < 0.0001 |
| | | EB | 8.1 | ± 0.5 | |
| | | A 0.5 | 7.6 | ± 0.4 | |
| | | A 1.0 | 8.1 | ± 0.4 | |
| | Males | DMSO | 5.6 | ± 0.3 | |
| | | EB | 5.6 | ± 0.4 | |
| | | A 0.5 | 6.9 | ± 0.4 | |
| | | A 1.0 | 6.1 | ± 0.4 | |
| Grooming (s) | Females | DMSO | 13.8 | ± 2.1 | F > M p < 0.003 |
| | | EB | 15.4 | ± 2.7 | |
| | | A 0.5 | 16.5 | ± 2.8 | |
| | | A 1.0 | 11.3 | ± 2.2 | |
| | Males | DMSO | 11.3 | ± 2.9 | |
| | | EB | 11.3 | ± 1.5 | |
| | | A 0.5 | 10.8 | ± 1.7 | |
| | | A 1.0 | 8.6 | ± 1.9 | |
| Rearing (s) | Females | DMSO | 20.5 | ± 5.2 | F > M p < 0.0001 |
| | | EB | 9.4 | ± 2.3 | |
| | | A 0.5 | 11.4 | ± 3.2 | |
| | | A 1.0 | 10.5 | ± 2.7 | |
| | Males | DMSO | 2.4 | ± 0.8 | |
| | | EB | 3.9 | ± 1.1 | |
| | | A 0.5 | 4.1 | ± 1.3 | |
| | | A 1.0 | 4.2 | ± 1.6 | |

Table 2.2: Sociability And Social Novelty Diagnostic Behaviors

Behaviors are shown that were computer-scored and used as diagnostic measures in the two social behavioral tests. Sex differences and direction of change are indicated for each behavior. P-values are provided when sexes were significantly different from one another. Data shown are mean + SEM.

## A. Sociability

| Measure | Sex | Treatment | Chamber | | | | Sex Diff. |
|---|---|---|---|---|---|---|---|
| | | | Social | | NonSocial | | |
| | | | Mean | SEM | Mean | SEM | |
| Time in proximity to stimulus animal (s) | Females | Vehicle | 221 | ± 17.7 | 82 | ± 7.6 | F = M |
| | | EB | 199 | ± 9.4 | 83 | ± 9.6 | |
| | | A1221 (0.5) | 201 | ± 18.5 | 68 | ± 6.8 | |
| | | A1221 (1) | 220 | ± 17.7 | 68 | ± 9.0 | |
| | Males | Vehicle | 218 | ± 19.7 | 45 | ± 7.0 | |
| | | EB | 216 | ± 16.4 | 52 | ± 5.5 | |
| | | A1221 (0.5) | 226 | ± 13.7 | 59 | ± 6.8 | |
| | | A1221 (1) | 262 | ± 25.6 | 34 | ± 4.7 | |
| Time spent investigating stimulus animal (s) | Females | Vehicle | 133 | ± 12.2 | 44 | ± 4.8 | F < M p < 0.004 |
| | | EB | 127 | ± 9.2 | 46 | ± 6.5 | |
| | | A1221 (0.5) | 113 | ± 10.2 | 35 | ± 4.6 | |
| | | A1221 (1) | 131 | ± 12.8 | 37 | ± 5.7 | |
| | Males | Vehicle | 144 | ± 16.1 | 23 | ± 3.8 | |
| | | EB | 148 | ± 12.5 | 28 | ± 3.5 | |
| | | A1221 (0.5) | 159 | ± 12.1 | 33 | ± 4.4 | |
| | | A1221 (1) | 167 | ± 21.5 | 16 | ± 3.0 | |
| Latency to investigate stimulus animal (s) | Females | Vehicle | 55 | ± 11.2 | N/A | N/A | F < M p < 0.005 |
| | | EB | 51 | ± 10.7 | N/A | N/A | |
| | | A1221 (0.5) | 48 | ± 48.5 | N/A | N/A | |
| | | A1221 (1) | 40 | ± 7.0 | N/A | N/A | |
| | Males | Vehicle | 128 | ± 34.1 | N/A | N/A | |
| | | EB | 78 | ± 26.2 | N/A | N/A | |
| | | A1221 (0.5) | 81 | ± 17.5 | N/A | N/A | |
| | | A1221 (1) | 76 | ± 26.5 | N/A | N/A | |
| Time spent nose touching (s) | Females | Vehicle | 7.6 | ± 1.0 | N/A | N/A | F < M p < 0.0001 |
| | | EB | 9.9 | ± 1.3 | N/A | N/A | |
| | | A1221 (0.5) | 7.6 | ± 1.2 | N/A | N/A | |
| | | A1221 (1) | 8.8 | ± 1.4 | N/A | N/A | |
| | Males | Vehicle | 13.4 | ± 2.3 | N/A | N/A | |
| | | EB | 11.1 | ± 1.4 | N/A | N/A | |
| | | A1221 (0.5) | 13.9 | ± 1.5 | N/A | N/A | |
| | | A1221 (1) | 12.9 | ± 2.0 | N/A | N/A | |
| Latency to first nose touch (s) | Females | Vehicle | 58 | ± 11.5 | N/A | N/A | F < M p < 0.001 |
| | | EB | 48 | ± 8.9 | N/A | N/A | |
| | | A1221 (0.5) | 65 | ± 13.8 | N/A | N/A | |
| | | A1221 (1) | 72 | ± 23.0 | N/A | N/A | |
| | Males | Vehicle | 133 | ± 34.0 | N/A | N/A | |
| | | EB | 79 | ± 25.6 | N/A | N/A | |
| | | A1221 (0.5) | 91 | ± 17.5 | N/A | N/A | |
| | | A1221 (1) | 90 | ± 30.2 | N/A | N/A | |

(Table 2.3 continued on next page)

## B. Social Novelty

| Measure | Sex | Treatment | Chamber | | | | Sex Diff. |
|---|---|---|---|---|---|---|---|
| | | | Familiar | | Novel | | |
| | | | Mean | SEM | Mean | SEM | |
| Time in proximity to stimulus animal (s) | Females | Vehicle | 96 | ± 8.7 | 147 | ± 11.9 | F < M p < 0.004 |
| | | EB | 97 | ± 12.6 | 146 | ± 14.1 | |
| | | A1221 (0.5) | 92 | ± 12.7 | 154 | ± 21.5 | |
| | | A1221 (1) | 110 | ± 12.3 | 129 | ± 12.6 | |
| | Males | Vehicle | 121 | ± 17.0 | 169 | ± 15.8 | |
| | | EB | 98 | ± 12.1 | 194 | ± 19.3 | |
| | | A1221 (0.5) | 107 | ± 12.1 | 138 | ± 13.0 | |
| | | A1221 (1) | 122 | ± 14.4 | 175 | ± 11.4 | |
| Time spent investigating stimulus animals (s) | Females | Vehicle | 54 | ± 6.5 | 74 | ± 9.2 | F < M p < 0.001 |
| | | EB | 50 | ± 5.5 | 76 | ± 8.2 | |
| | | A1221 (0.5) | 54 | ± 9.1 | 70 | ± 7.6 | |
| | | A1221 (1) | 57 | ± 7.2 | 68 | ± 8.7 | |
| | Males | Vehicle | 78 | ± 11.7 | 95 | ± 10.4 | |
| | | EB | 63 | ± 9.0 | 118 | ± 11.3 | |
| | | A1221 (0.5) | 66 | ± 8.4 | 87 | ± 9.2 | |
| | | A1221 (1) | 78 | ± 11.8 | 101 | ± 11.2 | |
| Time spent nose touching (s) | Females | Vehicle | 2.8 | ± 0.5 | 5.2 | ± 0.9 | F < M p < 0.001 |
| | | EB | 3.3 | ± 0.6 | 5.2 | ± 0.7 | |
| | | A1221 (0.5) | 2.9 | ± 0.5 | 4.4 | ± 0.8 | |
| | | A1221 (1) | 3.4 | ± 0.8 | 3.8 | ± 0.5 | |
| | Males | Vehicle | 4.4 | ± 1.0 | 10.1 | ± 1.7 | |
| | | EB | 5.7 | ± 1.3 | 10.4 | ± 1.1 | |
| | | A1221 (0.5) | 3.6 | ± 0.8 | 6.1 | ± 1.1 | |
| | | A1221 (1) | 3.7 | ± 0.8 | 7.8 | ± 1.1 | |
| Latency to first nose touch (s) | Females | Vehicle | 100 | ± 21.0 | 45 | ± 12.1 | F = M |
| | | EB | 138 | ± 25.9 | 41 | ± 18.3 | |
| | | A1221 (0.5) | 136 | ± 31.6 | 44 | ± 14.4 | |
| | | A1221 (1) | 109 | ± 25.0 | 38 | ± 19.4 | |
| | Males | Vehicle | 68 | ± 29.2 | 52 | ± 8.5 | |
| | | EB | 121 | ± 26.7 | 49 | ± 11.9 | |
| | | A1221 (0.5) | 170 | ± 33.1 | 52 | ± 14.0 | |
| | | A1221 (1) | 138 | ± 22.9 | 39 | ± 15.7 | |

Table 2.3: Sociability and Social Novelty Measures in Proximity to the Stimulus Cage

Behaviors are shown that were investigator-scored that took place in relationship to the stimulus animal, within at least one body length. Sex differences and direction of change are indicated for each behavior. P values are provided when sexes were significantly different from one another. Data shown are mean + SEM.

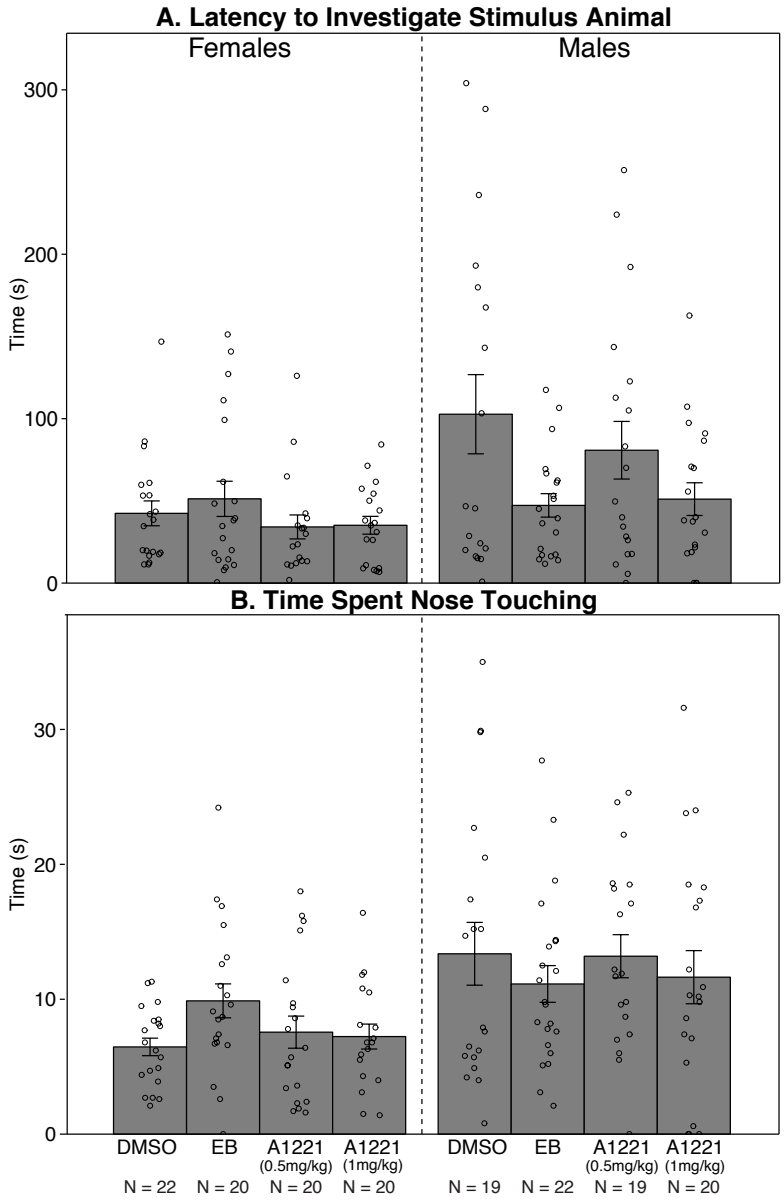



Figure 2.3: Sociability Test

Sociability test results are shown for the latency to investigate the stimulus animal (A), and the time spent nose touching (B). These measures were both sexually dimorphic, and higher in males than females ($p < 0.005$, 0.0001, respectively). However, no significant treatment effects within each sex were found. Data shown are mean $\pm$ standard error, with individual values shown as circles. N's are indicated below each bar.

**Social Novelty**

The computer-generated data for the diagnostic behaviors of the Social Novelty tests are summarized in Table 2.2B and investigator-scored data related to behaviors that took place in proximity to a stimulus animal are shown in Table 2.3B. Similar to the Sociability test, in the Social Novelty test there were many significant sex differences: females traveled a greater distance, were faster, and engaged in more grooming and rearing than males (Table 2.2B). Males spent more time investigating the stimulus animal and nose touching than females, as well as time in proximity to the stimulus rat. The sexes were equivalent in the latency to the first nose touch (Table 2.3B).

For the behaviors that took place in proximity to the stimulus animals, the dataset violated the assumptions for parametric analyses, despite any attempts at transformation. Student's t-test identified significant effects of sex for these behaviors (Table 2.3B), and overall the expected preference to engage in behaviors with a novel over a familiar animal was observed. Further analysis by Cohen's d effect size test revealed that this was altered in a sex- and dose-specific manner (Table 2.4). For the time spent in proximity to the stimulus animal (Figure 2.4A; Table 2.4A), three groups' effect sizes [females (A1221, 1 mg/kg) and males (DMSO; A1221, 0.5 mg/kg)] did not meet Cohen's d LARGE effect cut-off. For total time investigating the stimulus animal (Figure 2.4B; Table 2.4B), a LARGE effect size was only observed in EB males. Lastly, for time spent in direct nose-to-nose contact (Figure 2.4C; Table 2.4C), all non-vehicle female groups had a disrupted novelty preference; a LARGE effect size in the DMSO group was lost in the EB, and both A1221 groups. Among males, only the A1221 (0.5 mg/kg) group had an altered preference from DMSO. There was also an effect of treatment in the males, for which the A1221 (0.5 mg/kg) group spent significantly less time in direct nose-to-nose contact than both male control groups ($p < 0.05$; Figure 2.4C).

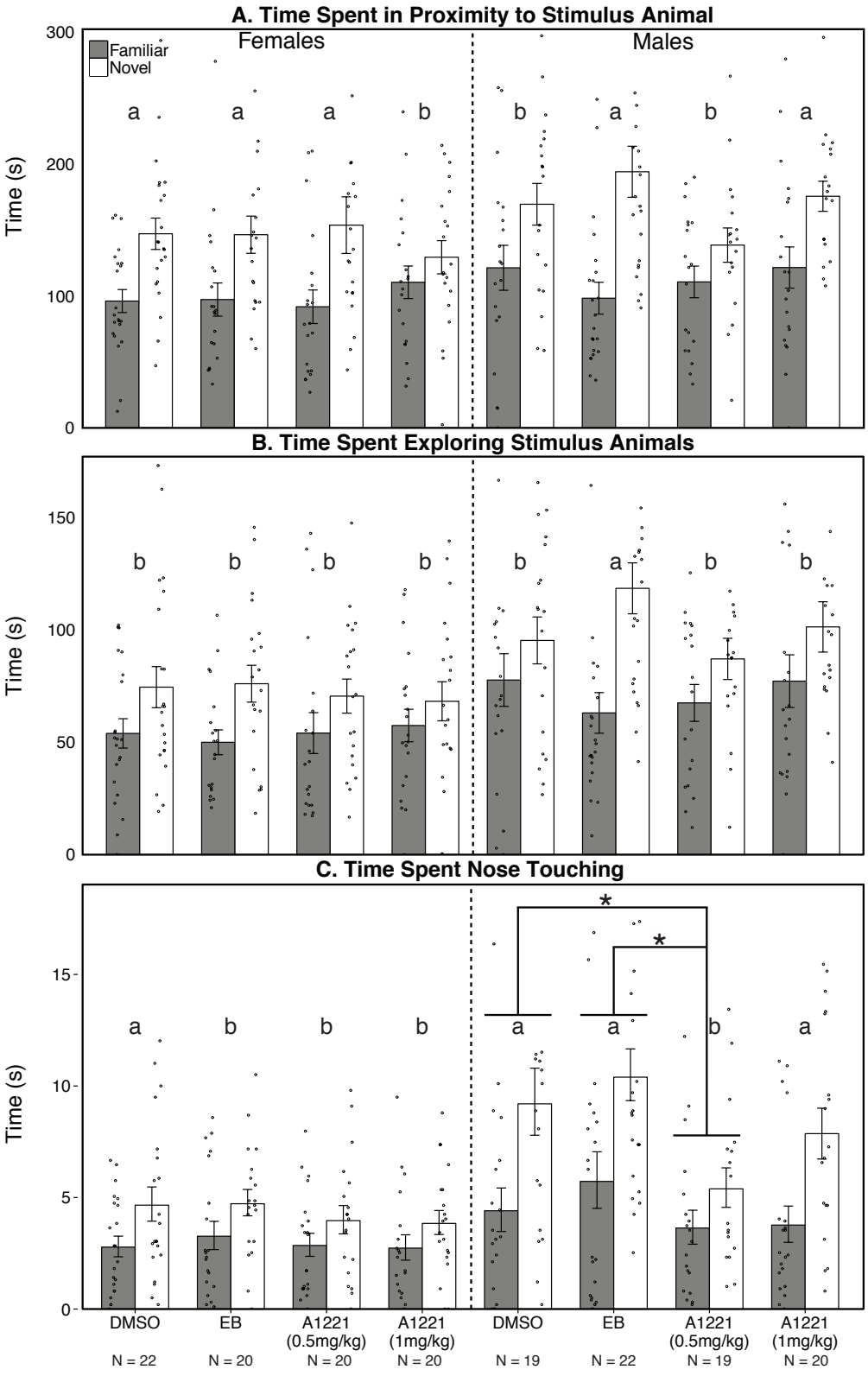



Figure 2.4: Social Novelty Test

Three measures involving social interactions during Social Novelty are shown for time spent in proximity to the stimulus animal (A), time exploring the stimulus animal (B), and time spent nose touching (C). In (A), for females the Cohen's d LARGE effect size was found for DMSO, EB, and A1221 (0.5 mg/kg), but not for A1221 (1 mg/kg). In males, the Cohen's d LARGE effect size was found for EB and A1221 (1 mg/kg) but not DMSO or A1221 (0.5 mg/kg). In (B) Cohen's d LARGE effect size was found only in the male EB group. In (C), in females, the Cohen's d LARGE effect size was found only in the DMSO group, whereas in males, the LARGE effect size was found in DMSO, EB, and A1221 (1 mg/kg) animals but not A1221 (0.5 mg/kg). In addition, total time spent nose touching was significantly different in the male A1221 (0.5 mg/kg) compared to male DMSO or EB groups ($p < 0.05$ for both). Data shown are mean $\pm$ standard error, with individual values shown as circles. *, $p < 0.05$. Groups with an identified Cohen's d LARGE effect size are indicated by a, and those without this effect by b. N's are indicated below the bars for each group and are the same for A, B, and C.

| A. Time in Proximity to Stimulus Animal | | | |
|---|---|---|---|
| **Females** | Cohen's d effect size | Percentile standing | Overlap (%) |
| DMSO | **1.0** | **84** | **62** |
| EB | **1.6** | **95** | **42** |
| A1221 (0.5) | **0.8** | **79** | **69** |
| A1221 (1) | 0.3 | 62 | 88 |
| **Males** | | | |
| DMSO | 0.7 | 76 | 73 |
| EB | **1.3** | **90** | **52** |
| A1221 (0.5) | 0.5 | 69 | 80 |
| A1221 (1) | **0.9** | **82** | **65** |

| B. Time Investigating Stimulus Animal | | | |
|---|---|---|---|
| **Females** | Cohen's d effect size | Percentile standing | Overlap (%) |
| DMSO | 0.7 | 76 | 73 |
| EB | 0.7 | 76 | 73 |
| A1221 (0.5) | 0.5 | 69 | 80 |
| A1221 (1) | 0.2 | 58 | 92 |
| **Males** | | | |
| DMSO | 0.2 | 58 | 92 |
| EB | **1.2** | **88** | **55** |
| A1221 (0.5) | 0.6 | 73 | 76 |
| A1221 (1) | 0.5 | 69 | 80 |

| C. Time Spent Nose Touching | | | |
|---|---|---|---|
| **Females** | Cohen's d effect size | Percentile standing | Overlap (%) |
| DMSO | **1.4** | **92** | **48** |
| EB | 0.5 | 69 | 80 |
| A1221 (0.5) | 0.4 | 66 | 84 |
| A1221 (1) | 0.5 | 69 | 80 |
| **Males** | | | |
| DMSO | **0.9** | **82** | **65** |
| EB | **0.8** | **79** | **69** |
| A1221 (0.5) | 0.5 | 69 | 80 |
| A1221 (1) | **1.0** | **84** | **62** |

Table 2.4: Cohen's d Effect Size for Behaviors

Cohen's d effect size was calculated for time spent in proximity to, investigating, and nose touching with, the familiar vs. the novel stimulus animal, in the Social Novelty test. An effect size of 0.8 or higher (in bold) is equivalent to Cohen's standard LARGE, which indicates that the mean of the control group (Familiar) is at the 79th percentile and sharing 69% overlap with the comparison group (Novel)

## DISCUSSION

This study tested effects of prenatal PCB exposures on suites of behaviors exhibited in tests of sociability and social novelty. Significant sex differences in these behaviors were observed. In the Sociability test, both sexes showed a strong preference for affiliating with a stimulus animal (vs. an empty cage), though in general, experimental males exhibited more interactions with the stimulus males than did experimental females with the stimulus females. In the Social Novelty test, there were several sexually dimorphic responses, but treatment resulted in few differences within each sex.

During the Sociability test, though the preference to spend time associating with the stimulus animal (vs the empty stimulus cage) was present in both sexes, the degree to which the animals interacted differed. Females were quicker to initiate contact with the stimulus animals than males; however, males spent more time investigating and interacting with the stimulus animal. While the increased interactions in male rats has been previously observed (Meaney and Stewart 1979), to our knowledge the reported differences in latency-to-investigation are novel. Consistent with the literature on social behavior in this particular paradigm (Choleris et al. 2006; Engelmann, Wotjak, and Landgraf 1995), there was a strong social preference, with the experimental animals spending more time in the chamber containing the stimulus animal than the chamber containing an empty stimulus cage.

During the Social Novelty test, sexual dimorphisms were again observed; males had longer latencies to initiate interactions, but ultimately interacted with both stimulus animals more so than did the females. Both sexes tended to associate and interact more with the novel conspecific compared to the familiar, in accordance with the literature

(Cox and Rissman 2011; Nadler et al. 2004; Wolstenholme et al. 2011). Our data suggest that (1) treatment does not alter the ability to acquire familiarity during the Sociability Stage, and (2) all animals are able to recognize and distinguish between the familiar and novel animal during the Social Novelty test.

Although most groups exhibited a strong novelty preference, there were exceptions, namely, the A1221 (1 mg/kg) female and the A1221 (0.5 mg/kg) male groups. This suggests that males and females have different sensitivities to A1221. Our laboratory has previously reported other sexually dimorphic changes due to gestational PCB exposure: only females have an increased postnatal body weight; only males exhibit an increased anogenital distance (Dickerson et al., 2011b; Walker et al., 2013). Developmental EDC exposure can also lead to sex-specific changes in behavior (Jacobsen et al., 2012; Kundakovic et al., 2013; Sobolewski et al., 2014; Williams et al., 2013).The male A1221 (0.5 mg/kg) group in our study also showed significantly less nose-to-nose interactions than vehicle males during Social Novelty, which was not seen in any of the female treatment groups. The degree of nose-touching that the A1221 (0.5 mg/kg) males displayed could be due to feminization of the brain areas involved in this behavior, as the behaviors were more similar to those observed in females. This also suggests that the males have an increased sensitivity of A1221-induced changes to adult social behaviors when compared to females. The EB groups were the only animals to lack the strong concordance of effect sizes when comparing the time spent in proximity and nose touching. EB animals in both sexes had the largest novelty preference in the time spent in proximity to the two stimulus animals but not in the nose-to-nose interactions, for which the female EB group lost the LARGE effect size comparison. This

suggests that EB treatment, regardless of sex, amplifies the tendency toward social approach, rather than interactive behavior.

Overall, the females showed significantly higher locomotor behaviors (average speed, distance, grooming, and rearing) than the males. The behaviors scored in proximity to the stimulus animals (time near the stimulus animal and cage, time investigating the stimulus animal and cage, time actively nose touching the stimulus animal), indicative of an interaction with the conspecific, were significantly higher in the males. When these same animals were presented with opposite sex stimulus animals during mate preference in a separate study, it was the males that displayed more approach than the females, the latter which were the more interactive sex (Topper, et al., 2014). This discrepancy illuminates how these interactions depend upon the context.

There was a sex- and dose-specific increase in the concentration of serum corticosterone in our experimental animals. Adrenal weights were heavier in females than in males, consistent with observations of Richter (1956) and our previous work using a transgenerational EDC (vinclozolin) exposure and evaluation of F3 descendants (Gillette et al., 2014). Circulating corticosterone concentrations were also higher in females relative to males. Within the females, the A1221 (0.5 mg/kg) group had a significantly higher concentration of this hormone, the latter unlikely to be due to a larger adrenal size as there were no treatment effects on this latter endpoint within each sex. This sex-specificity is parallel to a similar observation made for vinclozolin, another EDC that led to a female-only increase in corticosterone concentration in the F3 descendants (Gillette et al. 2014). Though A1221 is known to be weakly estrogenic, differences with the EB group in the present study suggests an alternate (non-estrogenic) mechanism. It is known

that the female rat is most sensitive to stress during proestrus (Viau and Meaney 1991); thus, the increase observed only in the A1221 (0.5 mg/kg) females may be due to an altered hormonal phenotype at the time of euthanasia, during which all females were in proestrus.

Previous studies indicate that prenatal exposure to Bisphenol A, diethylstilbestrol, and organophosphate insecticides result in changes in the social, anxiety, exploratory, and sex behavior (reviewed in Frye, 2014). However, many of the studies that found alterations in behavior lacked a positive control group. Using large sample sizes and a thorough characterization of the social behavioral phenotype in our experimental rats, we revealed relatively few differences between negative control (DMSO), positive control (EB), and two dosages of A1221. The sexually dimorphic nature of the changes observed, though few, demonstrate that the neurobiological mechanisms underlying the sex differences may present a means of varying vulnerabilities to the organizational processes leading to the acquisition of normal social behavior. This dichotomy must be accounted for when assessing the effects of any environmental toxicant.

# CHAPTER 3: IDENTIFYING THE EFFECTS OF AN EXTENDED GESTATIONAL EXPOSURE TO EDCS ON THE ADULT SOCIAL BEHAVIOR IN MALE AND FEMALE RATS


## ABSTRACT

Endocrine disrupting chemicals (EDCs) can lead to both temporary and permanent changes in physiology through diverse mechanisms by which exposure can perturb the hormonal system of an organism. EDC action during critical developmental periods could lead to long-lasting behavioral effects by disturbing the hormone-driven organization of the brain during gestation, which alters the activational response to endogenous hormones later in life. Polychlorinated biphenyls (PCBs) and Vinclozlin (VIN) are well-studied classes of EDCs that have been shown to act through estrogenic and anti-androgenic processes, respectively. Exposure to these compounds during gestation has been shown to have lasting effects on mating behaviors. Recently, gestational exposure to PCBs during late gestation have been found to elicit sex- and dose-specific changes in social interactions. The purpose of this experiment was to determine how exposure to these two EDCs during a n extended period of embryonic development may lead to alterations in the social behavior of male and female rats. Using the three-chamber apparatus, two tasks essential to the social behavior of rodents was assessed. While there was no effect of treatment on the preference of affiliate with a same-sex conspecific over an empty cage, males treated with VIN did not display the expected novelty preference when presented with a familiar and novel stimulus animal. We also observed several instances of exacerbated sex differences due to both PCB or VIN treatment. Overall, these data suggest that males are more susceptible to the effects of gestational exposure to VIN in the manifestation of adult social behavior.

## INTRODUCTION

Appropriate social behavior is an essential component of reproductive fitness. The processes governing the manifestation of normal adult behaviors are sexually dimorphic and established during gestation by hormone-sensitive organizational processes that govern brain development (Phoenix et al. 1959). Through activational processes induced by endogenous hormones during puberty, the manifestation of female- or male-typical adult social behaviors reflect the differences established during organization of the fetal brain (Arnold and Breedlove, 1985). This critical period of brain sexual differentiation is particularly sensitive to disruption via exposure to endocrine-disrupting chemicals (EDCs), as this life stage is characterized by differential exposures of the male and female brains to gonadal androgens and estrogens, perturbations of which change brain organization (Arnold and Breedlove, 1985).

Defined as an exogenous chemical or mixture of chemicals capable of interfering with any aspect of hormone function (Gore et al., 2015), EDCs are increasingly present in the environment. Though contamination of the food chain, inhalational, or occupational exposure, humans are exposed to potential EDCs on a regular basis (Reviewed by Frye et al. 2012). Of particular concern in the context of developmental neuroendocrinology are those EDCs capable of interfering with the systems governing the sexual differentiation of the brain during fetal development (Crews et al, 2006). Polychlorinated biphenyls (PCBs) and Vinclozolin (VIN) are two classes of EDCs shown to elicit changes in gene and protein expression, as well as alterations to sexual, anxiety, and social behaviors (Steinberg et al. 2008, Walker et al. 2014, Reilly et al. 2015, Gillette et al. 2017). Although not confined to any one particular hormonal pathway, the actions of PCBs and

VIN are at least partially mediated via estrogenic or anti-androgenic mechanisms, respectively (Jansen et al. 1993, Gray et al. 1994).

The present study aims to build upon literature of gestational exposure to these compounds can lead to functional alterations in the adult behavioral phenotype within a social setting. By observing both male and female rats exposed prenatally to EDCs, we tested the hypotheses that the sexes are differentially susceptible to disruption by different classes of EDCs. This study will also allow the ability to assess the influence of the duration of exposure, which encompasses a larger period of gestational development

## MATERIALS AND METHODS

### Animals and husbandry

This study was conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the university of Texas at Austin. Female and Male Sprague-Dawley rats were ordered in young adulthood (approximately 120 days old) from Harlan (Indianapolis, IN). Animals were housed in groups of 2-3 within standard clear polycarbonate rat cages and provided with a low phytoestrogen rat chow ad-libitum (Harlan-Tekland Extruded 2019 Global Rat Diet). For the duration of the experiment, the animals were maintained on a reverse 12:12 light cycle (lights on at 2300 h) and, following a one week habituation to the colony, handled weekly to familiarize the animals with tactile manipulation. Females were smeared daily for identification and recording of estrous cycle status. When a smear indicated proestrous, the females were paired with a sex-experienced male two hours after lights out and observed under red light for copulatory behaviors. If the female displayed receptive behaviors, the pair would

be left together overnight with food and water. A vaginal smear including sperm confirmed that mating took place and this event was deemed embryonic day 0 (E0). After a successful mating occurred, the male and female were separated and the pregnant female was single-housed in a cage for the duration of gestation. Nesting material was provided to the female several days prior to parturition on E21.

**Gestational Exposure to Endocrine Disrupting Chemicals**

Beginning on E8, pregnant dams were injected intraperitoneally at approximately 9:30am every morning for 11 days with the last injection taking place on E18. The three experimental treatment groups were as follows: Vehicle (6% dimethysulfoxide in sesame oil); Aroclor 1221 (A1221; a PCB mixture at a concentration of 1mg/kg; AccuStandard, New Haven, CT); and Vinclozolin (VIN; a fungicide at a concentration of 1mg/kg; Chem Service #N-13745, West Chester, PA). For the rest of the manuscript, the three treatment groups will be referred to as Vehicle, A1221, and VIN, respectively. E21 was the day of parturition, now deemed postnatal day 0 (P0). All F1 offspring were identified for sex, weighed, and anogenital distance measured to assist in culling the litter down to 5 pups from each sex in order to maintain a consistent sex ratio. The remaining 10 pups were returned to the dam's cage and monitored daily for somatic and sexual development. On P21 the pups were weaned into same-sex cages of 2-3 and provided with water and chow ad libitum. The weaned animals were weighed weekly and, beginning around P28 monitored for pubertal development (vaginal opening (VO) in females, preputial separation (PPS) in males). Following VO, females were smeared daily and cycle status recorder for the duration of their use in the experiment. Beginning ~ P90 the animals were placed through a battery of tests engaging their behavior within social, sexual, and

anxiogenic contexts. This report will focus on sociability and social novelty behavior, described below.

**Stimulus Animals and the Social Behavioral Testing Paradigm**

Stimulus Sprague-Dawley rats were bred within our colony. These animals were untreated with any chemicals. To avoid behavioral changes due to repeated testing, stimulus rats were not used for more than 3 trials in a day. Female stimulus animals were only used on diestrus, as indicated by vaginal smear.

A three-chamber apparatus (100 cm x 100 cm; Stoelting Co, Wood Dale, IL) was used as the testing arena for the two behavioral tasks (Moy 2004, Crews et al 2012, Reilly 2015). The testing area resembles an open field, separated into thirds. Doors in the central region of each wall allow an animal to pass from the central chamber to either lateral chamber, each containing a stimulus cage in the far corners. Behavioral testing occurred under dim red light during the dark phase of the animal's light cycle, approximately two hours following lights out. The experimental animal was placed in the central chamber to habituate to the apparatus for 5 minutes while the doors to the two lateral chambers were closed. Experimental females (F1 generation from the 3 treatment groups) were only run on days of diestrus, to avoid any confound of estrous cyclicity on behavioral outcomes. The same-sex stimulus animals used during testing were placed in a cylindrical cage (7 cm x 15 cm) located at the lower corner of the lateral chambers within the apparatus. Although the cage limited the movement of the stimulus animals throughout the test environment, direct interactions could take place across the bars of the cage. This enabled sniffing and nose-to-nose contact between the stimulus and experimental animals.

**Sociability**

Following the habituation period, the doors to the side chambers were lifted, allowing access to the two lateral chambers of the apparatus. For Sociability testing, only one of the stimulus cages contained a same-sex stimulus animal; the other chamber was empty. Placement of the stimulus rat on the left or right side was randomized. The experimental animal was allowed to roam freely in this configuration for 10 minutes while computer software (AnyMaze, Stoelting Co) tracked the experimental animal's position and video recorded the test. After 10 minutes, both the experimental and stimulus animals were removed from the apparatus and temporarily placed in their respective holding cages so that the apparatus could be dismantled and wiped clean with 70% ethanol prior to the next texting phase, which occurred immediately following Sociability.

**Social Novelty**

In the second test, the experimental rat was offered two stimuli: the same rat from Sociability (now familiar), and a novel, same-sex stimulus animal. Again, female stimulus rats were in diestrus. The two stimulus cages, each now containing a stimulus animal were randomly placed into the far corners of the testing arena. The experimental animal was returned to the center chamber and allowed to freely roam the entire apparatus for a 10-minute period, during which the same behaviors were recorded as in Sociability.

The AnyMaze computer software was used to track the animal's behavior during testing. Automatic measures were: total distance travelled (m) and average speed (m/s) throughout the apparatus. Being able to define discrete boundaries from within the

software, the program was also able to provide the time spent on each lateral side of the apparatus in addition to the time spent in proximity (defined as one body length away) from each stimulus cage. Video recordings of the tests were manually scored by an investigator blind to the sex or treatment of the animals. Investigator-scored behaviors were: (1) the active investigation of the stimulus cage (Time Spent Exploring Stimulus) or (2) instances when animals were in direct nose-to-nose contact (Time Spent Nose Touching). The latency (amount of time prior to the initial performance of each behavior) for the two investigator-scored behaviors was also included in analysis.

**Behavioral Analyses**

The measures used to analyze behaviors (defined in Table 3.1) were both computer- and investigator-scored. The former were considered for their diagnostic (Total Distance, Average speed) and descriptive (Time in each whole side: Social/Empty/Novel/Familiar/Center) qualities. Of these, one computer-scored measure (Time in Proximity to Stimulus Cage) and two investigator-scored measures (Time Spent Exploring Stimulus Cage and Time Spent Nose Touching) were evaluated in the context of the binary choice and a preference for one stimulus over another was defined at the group level through statistical analyses.

**Computer-Scored Behaviors**

| | | |
|---|---|---|
| Sociability | Total Distance | The total distance (m) travelled by the experimental animal for the entire duration of the 10 minute test |
| | Average Speed | The average speed of the experimental animal (m/s) for the duration of the experiment |
| | Time in Social Side | The total amount of time (s) an experimental animal spent on the side of the apparatus containing a stimulus cage holding a same-sex stimulus animal |
| | Time in Empty Side | The total amount of time (s) an experimental animal spent on the side of the apparatus contaning an empty stimulus cage |
| | Time in Center | The total amount of time (s) an experimental animal spent in the center of the apparatus, which held no stimulus cage |
| | Time in Proximity to Stimulus Cage* | The total amount of time (s) an experimental animal spent within one body-length of either stimulus cage |

| | | |
|---|---|---|
| Social Novelty | Total Distance | The total distance (m) travelled by the experimental animal for the entire duration of the 10 minute test |
| | Aerage Speed | The average speed of the experimental animal (m/s) for the duration of the experiment |
| | Time in Novel Side | The total amount of time (s) an experimental animal spent on the side of the apparatus containing a stimulus cage holding an unfamiliar same-sex stimulus animal |
| | Time in Familiar Side | The total amount of time (s) an experimental animal spent on the side of the apparatus contaning a stimulus cage holding the same stimulus animal from the previous test |
| | Time in Center | The total amount of time (s) an experimental animal spent in the center of the apparatus, which held no stimulus cage |
| | Time in Proximity to Stimulus Cage* | The total amount of time (s) an experimental animal spent within one body-length of either stimulus cage |

**Investigator-Scored Behaviors**

| | | |
|---|---|---|
| Sociability & Social Novelty | Latency to First Stimulus Explore Event* | The amount of time (s) prior to the first Stimulus Explore event |
| | Time Spent Exploring Stimulus Cage* | The amount of time (s) the experimental animal spent actively exploring the stimulus cage |
| | Latency to First Nose Touch Event* | The amount of time (s) prior to the first Nose Touch event |
| | Time Spent Nose Touching* | The amount of time (s) the experimental animal spent in direct nose-to-nose contact with the stimulus animal |

Table 3.1 Behaviors Quantified for Sociability and Social Novelty

A definition of the terminology used to describe the behavioral analysis of animals in Sociability and Social Novelty tests. Behaviors marked with an asterisk (*) indicate the measures of most salience for this paradigm., which were analyzed with respect to each stimulus option.

### Statistical analyses

For each measure, a one-way ANOVA was used to determine the effects of treatments within sex. To test sex differences within treatment, a t-test was used. To determine Social or Novel preferences, a paired t-test was used within each group. Data that did not meet the assumptions of normality or homogeneity of variance were analyzed with the appropriate non-parametric methods. All statistics are accompanied by effect size values (partial-$\eta p^2$, or $\varepsilon^2$ for ANOVA and Kruskal-Wallis tests, respectively; Cohen's d values for t-tests). Interpretation of the effect size values for partial-$\eta p^2$ and $\varepsilon^2$ were as follows: 0.01 = Small; 0.09 = Medium; 0.25 = Large). For Cohen's d: 0.2 = small; 0.5 = Medium; 0.8 = Large). Significance was set at $p < 0.05$.

## RESULTS

### Sociability test

In both sexes, there were no effects of treatment for any of the following measures (Table 3.2): Total distance, average speed, or total time in any of the three portions of the apparatus (social, empty, or center). In males, there was a trend toward significance ($p = 0.06$) regarding the amount of time each treatment group spent in the center chamber of the apparatus; this was associated with a Medium effect size. Regardless of treatment, there was a significant sex difference (F>M) in total distance and average speed, with a Cohen's d value indicative of a Large effect size (Table 3.2C). A significant sex difference was also found in the time A1221 or VIN, but not vehicle, animals spent in the portion of the chamber containing an empty stimulus cage (F>M). Lastly, the VIN animals were the only group to show a significant sex difference in the

time spent in the central chamber (M>F). All of these significant differences were associated with a Cohen's d value indicative of a Medium effect size (Table 3.2C).

***Time Spent in Proximity to Stimulus Cage***

Comparing the time spent within one body length of either stimulus cage, all groups spent significantly more time near the cage containing a stimulus rat compared to an empty cage (Figure 3.1A; $p < 0.05$ in vehicle males and females; $p < 0.0001$ in A1221 and VIN animals).

***Time Spent Exploring Stimulus and Latency to First Stimulus Explore Event***

No significant effects of treatment were observed in the latency to first exploration of a stimulus cage (Table 3.2), but significant sex differences in this behavior were observed in all treatment groups (M>F; Table 3.2C). Vehicle and A1221 sex differences were accompanied by a Large effect size, while the difference in VIN animals was met with a Medium Cohen's d value. There were no significant effects of treatment on the total time spent exploring the stimulus cages in either sex. Additionally, no sex differences in this behavior were observed. All treatment groups, regardless of sex, spent significantly ($p < 0.0001$) more time exploring the cage containing a stimulus animal compared to the empty stimulus cage (Figure 3.1B).

***Nose Touching And Latency to First Nose Touch Event***

In females, a significant effect of treatment was seen in the latency to the first Nose Touch event with a Medium effect size (Table 3.2A). Post hoc analyses indicated that this was driven by a significant difference between the VIN and A1221 females

(VIN<A1221; $p < 0.05$). No significant effects of treatment were seen in males. A significant sex difference in this measure was seen only in the VIN-treated animals (Medium effect size). No effects of treatment or sex were observed in the total time spent nose touching during the Sociability Test (Figure 3.1C).

A. Female Treatment Effects

| | Measure | Treatment | N | Mean | ± | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|---|
| Computer-Scored Measures | Total Distance (m) | Vehicle | 27 | 54.72 | ± | 1.62 | ANOVA | $F_{(3,83)} = 0.57$ | 0.57 | 0.01 |
| | | A1221 | 29 | 51.75 | ± | 2.03 | | | | |
| | | VIN | 30 | 54.07 | ± | 2.35 | | | | |
| | Average Speed (m/s) | Vehicle | 27 | 0.09 | ± | 0.004 | ANOVA | $F_{(3,83)} = 0.61$ | 0.55 | 0.01 |
| | | A1221 | 29 | 0.09 | ± | 0.003 | | | | |
| | | VIN | 30 | 0.09 | ± | 0.003 | | | | |
| | Time in Social Side (s) | Vehicle | 27 | 287.19 | ± | 11.96 | ANOVA | $F_{(3,84)} = 0.35$ | 0.70 | 0.01 |
| | | A1221 | 30 | 292.07 | ± | 8.21 | | | | |
| | | VIN | 30 | 300.19 | ± | 12.36 | | | | |
| | Time in Empty Side (s) | Vehicle | 27 | 212.80 | ± | 10.66 | ANOVA | $F_{(3,84)} = 0.76$ | 0.47 | 0.02 |
| | | A1221 | 30 | 207.48 | ± | 7.52 | | | | |
| | | VIN | 30 | 196.63 | ± | 10.01 | | | | |
| | Time in Center (s) | Vehicle | 27 | 99.67 | ± | 5.76 | ANOVA | $F_{(3,84)} = 0.13$ | 0.88 | 0.003 |
| | | A1221 | 30 | 100.04 | ± | 4.96 | | | | |
| | | VIN | 30 | 103.15 | ± | 5.34 | | | | |
| Investigator-Scored Measures | Latency to First Stimulus Explore Event | Vehicle | 27 | 15.19 | ± | 1.78 | KW | $X^2_{(3)} = 0.20$ | 0.91 | 0.003 |
| | | A1221 | 30 | 17.37 | ± | 3.01 | | | | |
| | | VIN | 30 | 15.82 | ± | 2.59 | | | | |
| | Time Spent Exploring Stimulus Cage | Vehicle | 27 | 148.56 | ± | 7.91 | KW | $X^2_{(3)} = 4.90$ | 0.08 | 0.06 |
| | | A1221 | 30 | 177.06 | ± | 11.86 | | | | |
| | | VIN | 30 | 148.93 | ± | 12.29 | | | | |
| | Latency to First Nose Touch Event | Vehicle | 27 | 72.83 | ± | 10.33 | KW | $X^2_{(3)} = 6.73$ | **0.03** | 0.08 |
| | | A1221 | 30 | 86.42 | ± | 13.30 | | | | |
| | | VIN | 30 | 46.74 | ± | 9.53 | | | | |
| | Time Spent Nose Touching | Vehicle | 27 | 15.42 | ± | 2.14 | KW | $X^2_{(3)} = 0.62$ | 0.73 | 0.01 |
| | | A1221 | 30 | 17.98 | ± | 3.01 | | | | |
| | | VIN | 30 | 14.50 | ± | 2.36 | | | | |

(Table 3.2 continued on next page)

B. Male Treatment Effects

| | Measure | Treatment | N | Mean | ± | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|---|
| Computer-Scored Measures | Total Distance (m) | Vehicle | 26 | 41.24 | ± | 3.89 | | | | |
| | | A1221 | 29 | 40.52 | ± | 2.46 | KW | $X^2_{(3)} = 0.92$ | 0.92 | 0.002 |
| | | VIN | 28 | 38.61 | ± | 2.82 | | | | |
| | Average Speed (m/s) | Vehicle | 26 | 0.07 | ± | 0.006 | | | | |
| | | A1221 | 29 | 0.07 | ± | 0.004 | ANOVA | $F_{(3,80)} = 0.20$ | 0.82 | 0.03 |
| | | VIN | 28 | 0.06 | ± | 0.005 | | | | |
| | Time in Social Side (s) | Vehicle | 29 | 286.00 | ± | 21.43 | | | | |
| | | A1221 | 29 | 313.15 | ± | 15.52 | ANOVA | $F_{(3,82)} = 0.56$ | 0.57 | 0.01 |
| | | VIN | 28 | 298.97 | ± | 17.07 | | | | |
| | Time in Empty Side (s) | Vehicle | 29 | 198.30 | ± | 20.20 | | | | |
| | | A1221 | 29 | 175.36 | ± | 13.55 | ANOVA | $F_{(3,82)} = 1.60$ | 0.21 | 0.04 |
| | | VIN | 28 | 158.92 | ± | 11.62 | | | | |
| | Time in Center (s) | Vehicle | 28 | 98.67 | ± | 6.19 | | | | |
| | | A1221 | 29 | 111.35 | ± | 7.25 | ANOVA | $F_{(3,81)} = 2.83$ | 0.06 | 0.07 |
| | | VIN | 27 | 124.65 | ± | 9.32 | | | | |
| Investigator-Scored Measures | Latency to First Stimulus Explore Event | Vehicle | 28 | 32.97 | ± | 5.37 | | | | |
| | | A1221 | 30 | 31.81 | ± | 3.81 | KW | $X^2_{(3)} = 1.00$ | 0.60 | 0.003 |
| | | VIN | 28 | 27.48 | ± | 3.86 | | | | |
| | Time Spent Exploring Stimulus Cage | Vehicle | 28 | 166.45 | ± | 14.21 | | | | |
| | | A1221 | 30 | 183.26 | ± | 6.74 | KW | $X^2_{(3)} = 4.61$ | 0.10 | 0.05 |
| | | VIN | 28 | 150.06 | ± | 12.16 | | | | |
| | Latency to First Nose Touch Event | Vehicle | 28 | 103.82 | ± | 21.62 | | | | |
| | | A1221 | 30 | 109.90 | ± | 19.76 | KW | $X^2_{(3)} = 0.07$ | 0.96 | 0.001 |
| | | VIN | 28 | 105.61 | ± | 19.42 | | | | |
| | Time Spent Nose Touching | Vehicle | 28 | 15.17 | ± | 1.93 | | | | |
| | | A1221 | 30 | 18.91 | ± | 2.18 | KW | $X^2_{(3)} = 2.42$ | 0.29 | 0.03 |
| | | VIN | 28 | 14.06 | ± | 2.18 | | | | |

(Table 3.2 continued on next page)

| C. Sex Effects within Treatment | | | | | | |
|---|---|---|---|---|---|---|
| | Measure | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| Computer-Scored Measures | Total Distance (m) | Vehicle | **0.003** | **0.88** | **Large** | **F>M** |
| | | A1221 | **0.001** | **1.36** | **Large** | **F>M** |
| | | VIN | **0.000** | **1.41** | **Large** | **F>M** |
| | Average Speed (m/s) | Vehicle | **0.003** | **1.44** | **Large** | **F>M** |
| | | A1221 | **0.001** | **1.43** | **Large** | **F>M** |
| | | VIN | **0.0001** | **1.57** | **Large** | **F>M** |
| | Time in Social Side (s) | Vehicle | 0.96 | 0.01 | Small | |
| | | A1221 | 0.24 | -0.32 | Small | |
| | | VIN | 0.10 | 0.04 | Small | |
| | Time in Empty Side (s) | Vehicle | 0.52 | 0.17 | Small | |
| | | A1221 | **0.04** | **0.55** | **Medium** | **F>M** |
| | | VIN | **0.02** | **0.66** | **Medium** | **F>M** |
| | Time in Center (s) | Vehicle | 0.91 | 0.03 | Small | |
| | | A1221 | 0.20 | -0.33 | Small | |
| | | VIN | **0.05** | **-0.53** | **Medium** | **M>F** |
| Investigator-Scored Measures | Latency to First Stimulus Explore Event | Vehicle | **0.004** | **-0.95** | **Large** | **M>F** |
| | | A1221 | **0.005** | **-0.80** | **Large** | **M>F** |
| | | VIN | **0.02** | **-0.72** | **Medium** | **M>F** |
| | Time Spent Exploring Stimulus Cage | Vehicle | 0.27 | -0.15 | Small | |
| | | A1221 | 0.65 | -0.12 | Small | |
| | | VIN | 0.94 | -0.02 | Small | |
| | Latency to First Nose Touch Event | Vehicle | 0.20 | -0.39 | Small | |
| | | A1221 | 0.32 | -0.26 | Small | |
| | | VIN | **0.01** | **-0.79** | **Medium** | **M>F** |
| | Time Spent Nose Touching | Vehicle | 0.93 | 0.02 | Small | |
| | | A1221 | 0.80 | -0.07 | Small | |
| | | VIN | 0.89 | 0.04 | Small | |

Table 3.2 Computer- and Investigator-Scored Measurements During the Sociability Test

A comprehensive report of the computer- and investigator scored measures during Sociability for females (A) and males (B). Sex differences are also shown (C). Data here and in Table 3 are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated. (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There was one significant effect of treatment in females in the Latency to First Nose Touch Event. There were numerous sex differences, all are indicated. F=female, M=male.

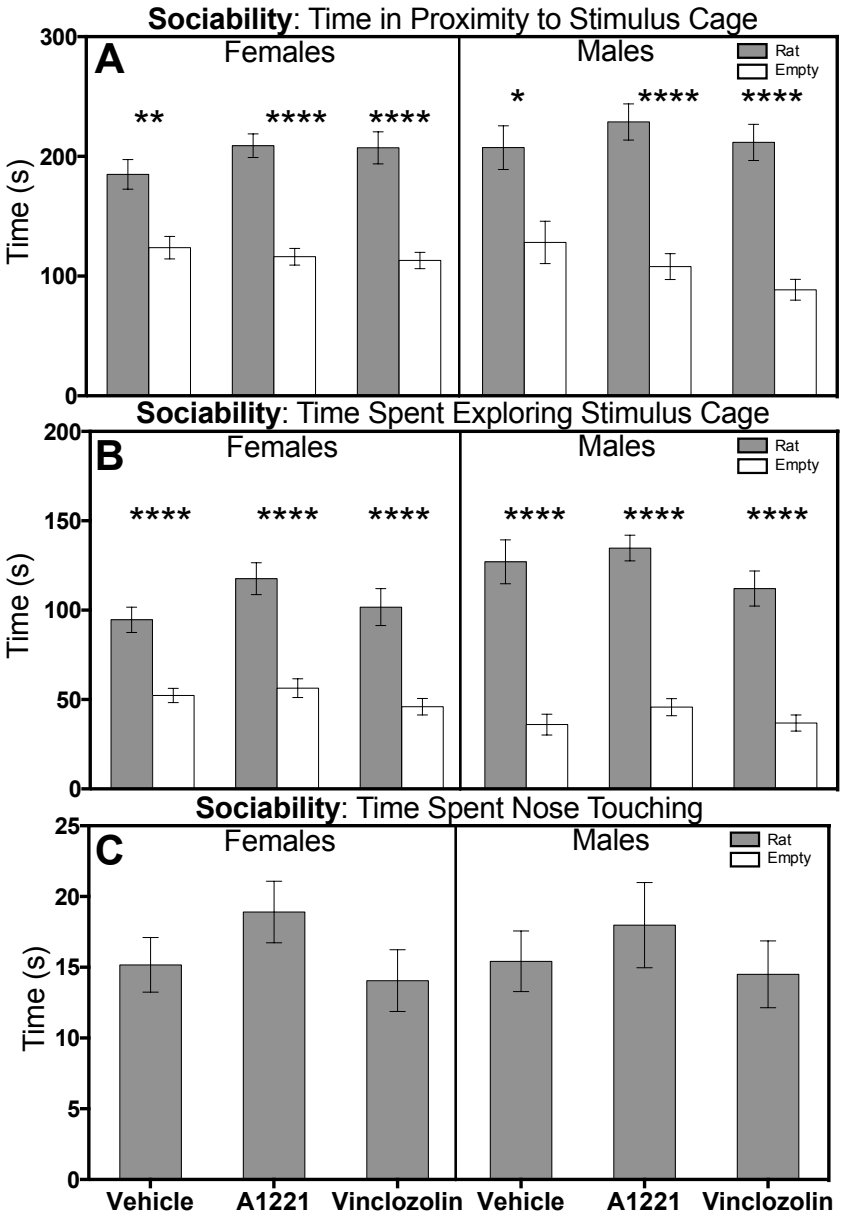



Figure 3.1: Sociability Test

Computer (A) and investigator-scored (B and C) measures of social exploration and interaction during the Sociability test. All groups spent significantly more time near (one body length away) the stimulus rat compared to the empty cage (A). All groups spent significantly more time exploring the stimulus cage containing the animal compared to the empty cage (B). There were no effects of treatment in the total time spent Nose Touching during this test (C). Mean and SEM are shown. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$

**Social Novelty test**

No significant effects of treatment were observed in the total distance travelled, average speed, or total time spent within any of the three chambers of the apparatus (table 3.3) . However, several treatment-specific sex differences were seen. In both total distance travelled and average speed, the Vehicle and A1221 treatment groups displayed a significant sex difference (F>M) that was not observed in the VIN groups (Table 3.3C). These differences in the Vehicle and A1221 groups were associated with Large and Medium effect sizes, respectively. The time spent in the chamber containing the Novel stimulus animal was sexually dimorphic only in the VIN group (F>M; Large effect size). Also unique to the VIN treatment group was a significant sex difference was seen in the time spent in the chamber containing the familiar stimulus animal (M>F; Medium effect size).

***Time Spent in Proximity to Stimulus Cage***

Comparing the time spent within one body length of either stimulus cage, the Vehicle and A1221 group, regardless of sex, spent significantly ($p < 0.01$) more time near the cage containing a Novel stimulus animal compared to the cage containing the Familiar animal (Figure 3.2A). In the VIN animals, only the females spent significantly more time near the novel animal.

***Time Spent Exploring Stimulus and Latency to First Stimulus Explore Event***

In both sexes, no effects of treatment were seen in the latency to the first stimulus explore event. Significant sex differences were seen in the A1221 and VIN group, but not the Vehicle. These differences were associated with Medium effect sizes. In the total time

spent exploring the stimulus cages, there were no effects of treatment or sex. Comparing the time spent exploring the Novel vs. Familiar stimulus animal, the VIN-treated males were the only group that did not spend significantly more time exploring the novel stimulus animal (Figure 3.2B).

***Nose Touching and Latency to First Nose Touch Event.***

No effect of treatment was seen in either sex in the latency to the first Nose Touch event or in the total time spent Nose Touching. Only the Vehicle-treated animals displayed a significant sex difference in these measures, both associated with a Large effect size. Comparing the sexes, only the Vehicle group had significant sex differences for Time Spent Nose Touching (F>M; Large effect size) and Latency to First Nose Touch Event (M>F; Large effect size). Comparing the time spend Nose Touching the Novel vs. Familiar stimulus animals, all groups except for the VIN males spent significantly more time Nose Touching the Novel stimulus animal compared to the Familiar (Figure 3.2C).

A. Female Treatment Effects

| | Measure | Treatment | N | Mean | ± | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|---|
| Computer-Scored Measures | Total Distance (m) | Vehicle | 27 | 52.43 | ± | 3.25 | KW | $X^2_{(3)} = 1.15$ | 0.56 | 0.01 |
| | | A1221 | 28 | 48.49 | ± | 1.99 | | | | |
| | | VIN | 30 | 46.67 | ± | 1.85 | | | | |
| | Average Speed (m/s) | Vehicle | 27 | 0.09 | ± | 0.01 | KW | $X^2_{(3)} = 1.17$ | 0.56 | 0.01 |
| | | A1221 | 28 | 0.08 | ± | 0.00 | | | | |
| | | VIN | 30 | 0.08 | ± | 0.00 | | | | |
| | Time in Novel Side (s) | Vehicle | 26 | 293.13 | ± | 10.33 | KW | $X^2_{(3)} = 1.75$ | 0.42 | 0.02 |
| | | A1221 | 30 | 287.92 | ± | 13.05 | | | | |
| | | VIN | 30 | 307.13 | ± | 10.17 | | | | |
| | Time in Familiar Side (s) | Vehicle | 27 | 196.46 | ± | 12.52 | ANOVA | $F_{(3,84)} = 1.85$ | 0.16 | 0.04 |
| | | A1221 | 30 | 192.49 | ± | 12.15 | | | | |
| | | VIN | 30 | 168.80 | ± | 8.14 | | | | |
| | Time in Center (s) | Vehicle | 27 | 117.02 | ± | 5.86 | ANOVA | $F_{(3,82)} = 0.16$ | 0.85 | 0.004 |
| | | A1221 | 29 | 113.49 | ± | 6.75 | | | | |
| | | VIN | 29 | 118.59 | ± | 7.08 | | | | |
| Investigator-Scored Measures | Latency to First Stimulus Explore Event | Vehicle | 27 | 8.83 | ± | 2.22 | KW | $X^2_{(3)} = 1.01$ | 0.60 | 0.01 |
| | | A1221 | 30 | 4.95 | ± | 0.78 | | | | |
| | | VIN | 30 | 5.40 | ± | 1.01 | | | | |
| | Time Spent Exploring Stimulus Cage | Vehicle | 27 | 151.03 | ± | 9.33 | ANOVA | $F_{(3,8e)} = 1.35$ | 0.26 | 0.04 |
| | | A1221 | 30 | 172.23 | ± | 8.73 | | | | |
| | | VIN | 30 | 168.09 | ± | 10.41 | | | | |
| | Latency to First Nose Touch Event | Vehicle | 27 | 17.59 | ± | 3.37 | KW | $X^2_{(3)} = 0.56$ | 0.76 | 0.01 |
| | | A1221 | 30 | 21.03 | ± | 3.70 | | | | |
| | | VIN | 30 | 19.26 | ± | 2.61 | | | | |
| | Time Spent Nose Touching | Vehicle | 27 | 19.78 | ± | 2.32 | KW | $X^2_{(3)} = 0.26$ | 0.87 | 0.00 |
| | | A1221 | 30 | 19.52 | ± | 2.58 | | | | |
| | | VIN | 30 | 18.98 | ± | 2.66 | | | | |

(Table 3.3 continued on next page)

B. Male Treatment Effects

| | Measure | Treatment | N | Mean | ± | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|---|
| Computer-Scored Measures | Total Distance (m) | Vehicle | 29 | 38.31 | ± | 3.46 | | | | |
| | | A1221 | 28 | 37.84 | ± | 3.33 | KW | $X^2_{(3)} = 1.46$ | 0.48 | 0.02 |
| | | VIN | 28 | 37.40 | ± | 5.12 | | | | |
| | Average Speed (m/s) | Vehicle | 29 | 0.06 | ± | 0.01 | | | | |
| | | A1221 | 28 | 0.06 | ± | 0.01 | KW | $X^2_{(3)} = 1.41$ | 0.50 | 0.02 |
| | | VIN | 28 | 0.06 | ± | 0.01 | | | | |
| | Time in Novel Side (s) | Vehicle | 28 | 283.45 | ± | 13.36 | | | | |
| | | A1221 | 30 | 283.07 | ± | 12.43 | KW | $X^2_{(3)} = 3.05$ | 0.22 | 0.04 |
| | | VIN | 28 | 247.28 | ± | 16.42 | | | | |
| | Time in Familiar Side (s) | Vehicle | 29 | 199.36 | ± | 10.70 | | | | |
| | | A1221 | 30 | 210.43 | ± | 13.74 | KW | $X^2_{(3)} = 0.24$ | 0.88 | 0.00 |
| | | VIN | 27 | 211.91 | ± | 13.11 | | | | |
| | Time in Center (s) | Vehicle | 28 | 116.53 | ± | 8.29 | | | | |
| | | A1221 | 30 | 106.19 | ± | 8.85 | KW | $X^2_{(3)} = 3.10$ | 0.21 | 0.04 |
| | | VIN | 28 | 126.80 | ± | 12.01 | | | | |
| Investigator-Scored Measures | Latency to First Stimulus Explore Event | Vehicle | 28 | 9.96 | ± | 2.68 | | | | |
| | | A1221 | 30 | 13.35 | ± | 4.24 | KW | $X^2_{(3)} = 0.21$ | 0.90 | 0.002 |
| | | VIN | 28 | 12.21 | ± | 2.90 | | | | |
| | Time Spent Exploring Stimulus Cage | Vehicle | 28 | 147.38 | ± | 9.38 | | | | |
| | | A1221 | 30 | 166.61 | ± | 8.41 | KW | $X^2_{(3)} = 3.63$ | 0.16 | 0.04 |
| | | VIN | 28 | 165.51 | ± | 9.87 | | | | |
| | Latency to First Nose Touch Event | Vehicle | 28 | 34.36 | ± | 5.17 | | | | |
| | | A1221 | 30 | 37.32 | ± | 7.44 | KW | $X^2_{(3)} = 0.78$ | 0.68 | 0.01 |
| | | VIN | 28 | 27.63 | ± | 4.01 | | | | |
| | Time Spent Nose Touching | Vehicle | 28 | 11.42 | ± | 1.47 | | | | |
| | | A1221 | 30 | 15.39 | ± | 1.80 | KW | $X^2_{(3)} = 2.11$ | 0.35 | 0.02 |
| | | VIN | 28 | 15.79 | ± | 2.15 | | | | |

(Table 3.3 continued on next page)

| C. Sex Effects within Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Measure | | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| Computer-Scored Measures | Total Distance (m) | Vehicle | **0.004** | **0.82** | **Large** | **F>M** |
| | | A1221 | **0.01** | **0.72** | **Medium** | **F>M** |
| | | VIN | 0.09 | 0.44 | Small | |
| | Average Speed (m/s) | Vehicle | **0.004** | **0.99** | **Large** | **F>M** |
| | | A1221 | **0.01** | **0.76** | **Medium** | **F>M** |
| | | VIN | 0.10 | 0.44 | Small | |
| | Time in Novel Side (s) | Vehicle | 0.56 | 0.16 | Small | |
| | | A1221 | 0.78 | 0.06 | Small | |
| | | VIN | **0.003** | **0.83** | **Large** | **F>M** |
| | Time in Familiar Side (s) | Vehicle | 0.86 | -0.05 | Small | |
| | | A1221 | 0.33 | -0.25 | Small | |
| | | VIN | **0.01** | **-0.75** | **Medium** | **M>F** |
| | Time in Center (s) | Vehicle | 0.96 | 0.03 | Small | |
| | | A1221 | 0.51 | 0.16 | Small | |
| | | VIN | 0.55 | -0.15 | Small | |
| Investigator-Scored Measures | Latency to First Stimulus Explore Event | Vehicle | 0.75 | -0.10 | Small | |
| | | A1221 | **0.03** | **-0.54** | **Medium** | **M>F** |
| | | VIN | **0.03** | **-0.65** | **Medium** | **M>F** |
| | Time Spent Exploring Stimulus Cage | Vehicle | 0.78 | 0.02 | Small | |
| | | A1221 | 0.64 | 0.16 | Small | |
| | | VIN | 0.86 | 0.05 | Small | |
| | Latency to First Nose Touch Event | Vehicle | **0.01** | **-0.87** | **Large** | **M>F** |
| | | A1221 | 0.06 | -0.55 | Medium | |
| | | VIN | 0.08 | -0.52 | Medium | |
| | Time Spent Nose Touching | Vehicle | **0.004** | **0.91** | **Large** | **F>M** |
| | | A1221 | 0.19 | 0.35 | Small | |
| | | VIN | 0.35 | 0.27 | Small | |

Table 3.3: Computer- and Investigator-Scored Measurements During the Social Novelty Test

A comprehensive report of the computer- and investigator scored measures during Social Novelty for females (A) and males (B). Sex differences are also shown (C). Data here and in Table 2 are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated. (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There were no effects of treatment. There were numerous sex differences, all are indicated. F=female, M=male.

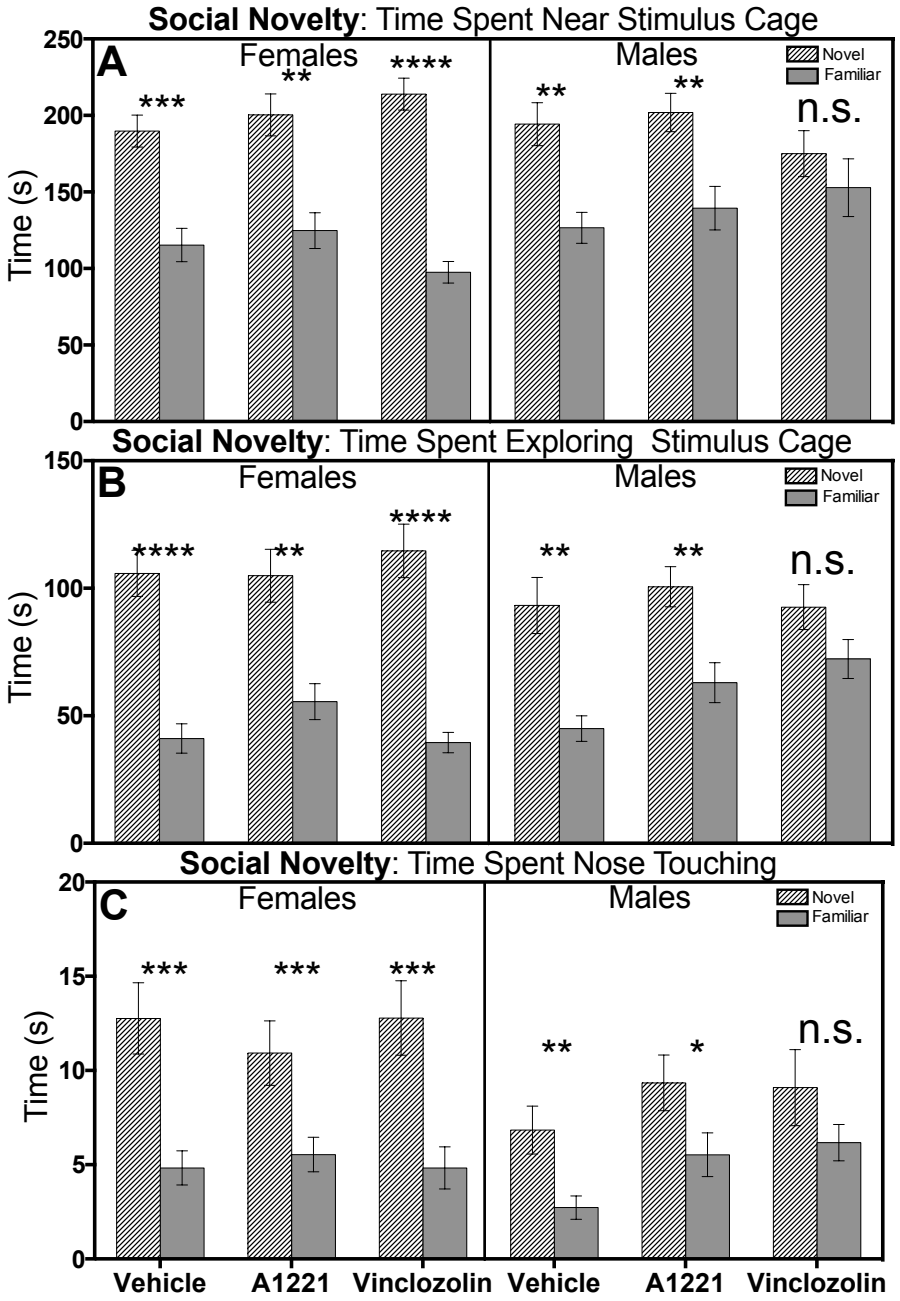



Figure 3.2: Social Novelty Test

Computer (A) and investigator-scored (B and C) measures of social exploration and interaction during the Sociability test. All groups except for the VIN males spent significantly more time in proximity to (A) exploring (B) and nose touching (C) the stimulus cage containing a novel stimulus animal compared to the familiar. Mean and SEM are shown. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$.

## DISCUSSION

The aim of this experiment was to determine the behavioral implications of gestational exposure to the polychlorinated biphenyl mixture (A1221) or the fungicide Vinclozolin (VIN), two compounds classified as endocrine-disrupting chemicals. With an exposure paradigm that encompasses the beginning of the hormone-sensitive period of sexual differentiation (Breedlove et al. 1992, Rees at al. 1990, Wagner et al. 1998), we determined how interfering with the developmental processes in the fetus leads to life-long changes in the sexually-dimorphic adult social phenotype. Overall, we found in a test that gauges the animal's affiliative behavior by providing a choice between a stimulus rat and an empty cage, all groups, regardless of treatment, displayed a clear-cut preference for the stimulus animal - a phenotype expected in this species (Engelmann et al. 1995, Moy et al. 2004, Choleris et al. 2006, Crews et al. 2012, Reilly et al. 2015). When presented with a familiar vs. an unknown stimulus animal, a subject rat will typically spend more time associating with the novel conspecific (Nadler et al. 2004, Cox and Rissman 2011, Wolstenholme et al. 2011). This task tests the subject's ability to discriminate between, to remember, and/or to demonstrate a preference for, a novel or familiar rat. Here, all groups except for the males prenatally treated with VIN displayed the expected preference for the Novel conspecific. Therefore, while there do not appear to be effects of A1221 or VIN on the affinity to engage in social behaviors, gestational exposure to VIN has a sex-specific influence. There are several non-mutually exclusive explanations for this outcome: VIN may have changed an animal's capacity to discriminate one stimulus from another, abolished the preference for a novel animal, or affected social memory.

**Effects of EDCs on Social Behaviors**

Our results showed that for both the Sociability and the Social Novelty tests, there were no effects of treatment on the total distance travelled or average speed, indicating that these treatments had no effects on the locomotive capabilities of these animals. This finding is consistent with previous studies looking at this outcome in rats (Reilly et al. 2015, Gillette et al. 2017). Aside from a trend ($p = 0.06$) toward significance in the time that males spent in the central portion of the chamber during Sociability, there were no effects of treatment in the overall time spent in the Social or Empty chambers during Sociability. During Social Novelty, there were no effects of treatment on the overall time spent in the Novel, Familiar, or Center chambers. By comparison, those measures that required close-in interactions (Stimulus Explore and Nose Touching) revealed some treatment effects. For Social Novelty, there was a significant effect of treatment in females on the latency to initiate the first Nose Touch event where VIN females performed this behavior faster than females treated with A1221.

In the test of Sociability, in accordance with previous studies on the effects of PCBs in the social behaviors of the animals (Reilly et al. 2015, Bell et al. 2015), there were no alterations of preference, with all groups to spend more time investigating and interacting with the stimulus cage holding an animal compared to the empty cage. Studies with PCBs that have shown differences in an animal's preference for a social vs. non-social stimuli did not use A1221, but a mixture of congeners A1247 and A1277 (Jolous-Jamshidi et al. 2010). During Social Novelty, most groups, with the exception of the VIN males, spent more time associating and interacting with the novel stimulus animal, the

expected choice. Similarly, for other measures of interaction (Time Near, Stimulus Exploration, and Nose Touching) the VIN males did not display the expected preference. This adds to a growing body of literature which implicates EDC exposure to an alteration of social behaviors. (PCBs: Jolous-Jamshidi et al. 2010, Reilly et al. 2015; BPA: Adriani et al. 2003, Wolstenholme et al. 2013, Pthalates: Wang et al. 2016).

**Sex Differences in EDC Effects**

The behaviors selected for this study are sexually dimorphic; EDCs abolished some sex differences and introduced others. Vinclozolin treatment led to the most disruptions in this regard. Four sex differences found in Vehicle animals were abolished in VIN-treated animals. VIN treatment also induced 7 instances of sex differences not seen in Vehicle animals. . For A1221, 4 sex differences were altered compared to Vehicle (2 abolished, 2 induced). This corroborates the hypothesis that the gestational processes that differentially organize the sexes are subject to changes by exposure to EDCs. A previous study in rats treated with A1221 using the same 3-chambered apparatus (Reilly et al. 2015), albeit using a shorter exposure time (dams were treated on E16 and E18 only, compared to the present study when treatment was from E8-E18) showed several sex differences that were not present here. Most surprising was the absent, or in some cases reversed, sex difference (M>F) in the amount of time spent Nose Touching the stimulus animals. That prior study also used gonadectomized stimulus animals that were untreated with hormone whereas the current study utilized intact rats (with female stimulus rats used on diestrus); the differences in stimuli could certainly affect their salience and the motivation for the experimental rats to interact.

**Conclusions and Further Research Needs**

The results of this experiment indicated that the behavioral phenotype of the sexes is differentially susceptible to the actions of gestational EDC exposure. Through the use of the three-chamber test, we provide evidence that gestational exposure to the PCB mixture A1221 or the fungicide Vinclozolin did not alter an adult rat's social motivation, as tested during Sociability. The results of Social Novelty suggest that gestational Vinclozolin, but not A1221, led to sex-specific alterations of the brain, particularly in males, that that was demonstrated by functional differences in social novelty preference. The lack of preference shown in these animals may due to an inability to discriminate; alternatively, the animals may be capable of distinguishing a novel vs. a familiar rat, with treatment altering the preference toward the familiar animal. Finally, there may be deficits in social memory. Further experimentation into the motivations underlying an animal's penchant for proximity is needed.

In addition to the behavioral effects of VIN, we failed to observe any changes in the Novelty Preference of A1221 in either sex. It was previously reported at A1221 led to a sex- and dose-specific disruption of the Novelty Preference phenotype (0.5 mg/kg and 1.0 mg/kg dosages in females, and 0.5 mg/kg dose in males). Here, the A1221 animals (all 1.0 mg/kg) were similar to vehicle.

Further experimentation on the mechanisms and brain regions underlying social behavior in these animals will provide insight into the way that fetal PCBs or VIN may lead to the phenotypes observed. Specific neural circuits have been implicated in the processes governing normal social functioning; in particular, the nonapeptides oxytocin and vasopressin are crucial in the regulation of anxiety, social, and sex behavior, and are

steroid-sensitive (Bale et al. 2001, Bielsky et al. 2004, Egashira et al. 2007, Young et al. 2006, Veenema et al. 2013). Future work will focus on how EDCs sculpt the development of these neuropeptide systems.

# CHAPTER 4: APPLICATION OF A NOVEL SOCIAL CHOICE PARADIGM TO ASSESS EFFECTS OF PRENATAL ENDOCRINE-DISRUPTING CHEMICAL EXPOSURE IN RATS

The text in this section is excerpted from Reilly MP, Weeks CD, Crews D, and Gore AC. Journal of Comparative Psychology (2018), with permission from the journal. As first author, I was involved with all of the experimentation, analysis, and preparation of the manuscript.


## ABSTRACT

Endocrine disrupting chemicals (EDCs) exposures during critical periods of gestation cause long-lasting behavioral effects, presumably by disturbing the hormone-driven organization of the brain. Among such EDCs are polychlorinated biphenyls (PCBs), a class of industrial chemicals. PCB exposure in utero lead to alterations in mating behaviors and other sexually-dimorphic social interactions in rats. Many of the previous studies on social behavior give the experimental animal a single or binary choice. This study applies a more complex behavioral apparatus. Using a X-shaped Plexiglas apparatus (FourPlex) the experimental animal is able to distinguish and choose among stimulus animals of the same or opposite sex, and of different hormonal status. Behavioral choices were affected by the sex of the experimental rat, but there was little evidence that prenatal exposure to PCB affected the decision-making processes. This may be due to the relatively low levels and short duration of the EDC used. Importantly, the results differ from our prior results of a simple binary choice model, showing that how an animal behaves in a more complex social paradigm does not predict the outcome in a simple choice model, and vice versa.


## INTRODUCTION

Sociality is a complex trait; it arises from the qualities within an individual and the social interactions between individuals. The spectrum of social systems is enormous,

from animals that live in total isolation except to mate, and others that live in groups from dyads to thousands. To reduce this complexity in the laboratory setting, behavioral neuroscientists have most commonly taken the reductionist approach. For laboratory rodents, most tests comprise a two-choice paradigm, in which an animal is given a choice between an empty compartment and a stimulus animal, or between two animals that differ in some way (e.g., male vs. female; hormone-primed vs. unprimed; females in different stages of receptivity; etc.). Exceptions have been the socioecological work pioneered by RD Lisk and M McClintock in their laboratory studies of hamsters and rats in large enclosures (Huck, Lisk, & Gore, 1985; McClintock, 1987). More recently, D Kimchi and J Curley independently developed methods of quantifying social interactions within groups of mice (So, Franks, Lim, & Curley, 2015; Weissbrod et al., 2013). Other labs have adopted a multiple-choice system in social models of mate choice (Ferreira-Nuño, Morales-Otal, Paredes, & Velázquez-Moctezuma, 2005). Results of these studies challenge the premise that results from a two-choice paradigm can be extrapolated to more complex interactions; yet the two-choice paradigm continues to be the norm.

Using behavior as a biomarker, our overall goal was to characterize the effects of prenatal exposure to ecologically-relevant levels of a class of endocrine-disrupting chemicals (EDCs), polychlorinated biphenyls (PCBs). Humans and wildlife continue to have detectable levels of PCBs in body tissues, despite the ban of these chemicals in developed countries in the 1970s (Gladen, Doucet, & Hansen, 2003). PCBs are persistent, are in the food chain, and can be transported around the world by air and water currents, as well as by migratory species that feed in contaminated areas for part of the year (Crews & Gore, 2011). Previous experimental work from our lab and others on rodents has shown that the brain is sensitive to environmental contamination, especially when exposure occurs during critical periods of development. One such life stage is that of

brain organization, when gonadal steroid hormones exert influences on the developing nervous system in a sex-typical manner, setting the stage for subsequent pubertal hormones to activate these neural pathways. This enables the manifestation of sexually dimorphic behaviors involved in reproduction (McCarthy & Arnold, 2011).

Exposures to PCBs during periods of organization and/or activation perturb adult reproductive physiology and behavior (Dickerson, Cunningham, & Gore, 2011; Steinberg, Walker, Juenger, Woller, & Gore, 2008; Walker, Goetz, & Gore, 2013). While other sexually-dimorphic social behaviors have also been investigated for effects of developmental EDC exposures, this research has exclusively relied upon two-choice systems (Belloni et al., 2011; Crews et al., 2012; Gillette et al., 2014; Reilly et al., 2015; Venerosi, Ricceri, Tait, & Calamandrei, 2012; Wolstenholme et al., 2011). However, mate and social choice in naturalistic settings are complex processes that involve, among other things, the complementarity of the chooser and the chosen, with the mutual evaluation of qualities related to a mate's fitness (Crews, 2010; Carson, 2003). In the mate choice literature, the terms appetitive and consummatory are used to describe exploratory/proceptive behaviors prior to mating, and the act of coitus itself, respectively. These concepts that can be extrapolated to more complex social settings where conspecifics are first evaluated (i.e., prosocial behaviors) and subsequently decide to engage in or avoid interactions. Here, we sought to develop and validate a unique four-choice paradigm, referred to as the FourPlex, that retains the basic choice aspect but allows for a greater variety of social stimuli to better model the types of interactions encountered in nature. The stimulus rats were opposite- and same-sex conspecifics of differing hormonal status, and results showed that when this complexity was added, these clear choices of individuals were quite different from those observed in the dyadic situation.

## MATERIALS AND METHODS

### Animals and Husbandry

Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN), and all animal procedures were conducted in compliance with a protocol (AUP-2013-00054) approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin, following the guidelines from the NIH. All animals were housed in a colony room with controlled temperature (22 C) and light cycle (12:12 dark:light, lights on at 2400). Rats were fed a low-phytoestrogen diet (Harlan, Indianapolis, IN Cat. # 2019) available ad libitum. Virgin females were mated with sexually experienced males. The day following successful mating, as indicated by a sperm-positive vaginal smear, was termed embryonic day 1 (E1).

### Gestational Exposure Paradigm

Following confirmed pregnancy, dams were exposed to one of four treatments, administered via intraperitoneal injections, on E16 and E18, the beginning of the period of brain sexual differentiation (Davis, Popper, & Gorski, 1996; Jacobson, Shryne, Shapiro, & Gorski, 1980). The experimental treatment groups were as follows: (1) Vehicle [Negative control - 3% dimethylsulfoxide (DMSO) in sesame oil]; (2) Estradiol benzoate [(EB) 50 μg/kg – positive control for the estrogenic effects of PCBs)]; (3) the PCB mixture, Aroclor 1221 (A1221, 0.5 mg/kg), or (4) A1221 (1 mg/kg). We henceforward refer to these groups as vehicle, EB, A1221 (0.5) and A1221 (1.0), respectively, as summarized in Table 4.1. Selected treatments and dosages were based on prior work conducted in the Gore lab that showed physiological, behavioral, and neuroendocrine effects that were manifested in a sex- and developmental age-specific manner; however, these dosages caused no overt toxicity or pregnancy complications

(Dickerson et al., 2011; Steinberg, Juenger, & Gore, 2007; Steinberg et al., 2008; Topper, Walker, & Gore, 2015; Walker et al., 2013, Gillette et al., 2014; Reilly et al. 2015). The number of litters per treatment was 9, 10, 10, and 10, respectively. Although we did not measure body burden or tissue content in the exposed offspring, the literature suggests that maternal-fetal transfer results in a body burden of approximately 1-2 μg/kg A1221, and 100 ng/kg EB in the fetuses (Takagi, Aburada, Hashimoto, & Kitaura, 1986). This falls in the approximate range of human exposures (DeKoning & Karmaus, 2000; Lin, Pessah, & Puschner, 2013).

The day of parturition was postnatal day 0 (P0). The day after birth, P1, the pups were weighed and their anogenital distance measured; litters were culled to 4 males and 4 females. The pups were monitored daily for age at eye opening, and body weight and anogenital distance was measured and recorded weekly from birth to weaning, which occurred at P21. Once rehoused in same-sex groups, rats were monitored daily for signs of pubertal development: vaginal opening in females and preputial separation in males (Steinberg et al., 2007; Walker, Kirson, Perez, & Gore, 2012). In females, beginning on the day of vaginal opening, daily vaginal smears were taken and cell cytology was examined as a measure of estrous cyclicity in the females. Weekly body weights continued to be recorded for all animals throughout their lifetimes. There were no effects of prenatal treatment on litter size or sex ratio, age at puberty, or estrous cyclicity (data not shown), as previously published (Gillette et al., 2017; Reilly et al., 2015).

| Compound | Group name | Function |
|---|---|---|
| | | |
| Dimethylsulfoxide (DMSO) 3% in sesame oil | Vehicle | Negative control |
| Estradiol benzoate (EB) 50 µg/kg, dissolved in vehicle | EB | Positive estrogenic control |
| Aroclor 1221 (A1221) 0.5 mg/kg, dissolved in vehicle | A1221 (0.5) | Experimental PCB, lower dosage |
| Aroclor 1221 (A1221) 1.0 mg/kg, dissolved in vehicle | A1221 (1.0) | Experimental PCB, higher dosage |

Table 4.1. Treatment Group Terminology

Pregnant rats were injected subcutaneously in late gestations (embryonic days 16 and 18) with the above treatments. Prenatally exposed rats were the experimental subjects, tested in the FourPlex. Experimental groups are referred to by the group names.

**Stimulus Rats**

Ten male and 10 female stimulus rats (Sprague-Dawley) were purchased as young adults from Harlan and gonadectomized under isoflurane anesthesia. After ovariectomy (OVX), females were implanted subcutaneously, in the nape of the neck, with a Silastic capsule (1.98mm I.D. × 3.18mm O.D. × 5-mm length; Dow Corning Corporation, Midland, MI). Five females received capsules packed with 5% crystalline 17β-estradiol (Cat. #: E8875; Sigma-Aldrich, St. Louis, MO) with 95% cholesterol; and 5 females received a 100% cholesterol capsule (Wu et al., 2010). Five males received capsules containing 100% testosterone (Cat. #: T1500; Sigma-Aldrich, St. Louis, MO) and 5 received cholesterol capsules (Wu et al., 2010). This resulted in 4 categories of stimulus rats (n = 5 per category) as described below. Stimulus animals were untreated with EDCs or their controls.

## Behavioral Testing Paradigm

### *Behavioral Testing*

Beginning when experimental (EDC or vehicle-treated) rats were aged P56, one male and one female from each litter was used for testing. The total number of behaviorally characterized animals was 39 females and 39 males. Females were only used on diestrus to remove the possible confound of estrous cycle status on behavioral outcomes. Testing was conducted under dim red light during the dark period of their light-dark cycle, beginning approximately two hours following lights out. Between animals, the behavioral apparatus was thoroughly cleaned with a 70% ethanol solution.

### *Apparatus*

The FourPlex apparatus was designed to reveal the ability of the experimental animal to discriminate between, and preferentially associate with, four possible stimulus partners (Figure 4.1). Four opaque Plexiglas inserts were constructed and placed inside of a 100 cm x 100 cm apparatus (Stoelting), resulting in an X-shaped testing arena. Each of the four arms held a stimulus cage in the far corner of the apparatus. For the purposes of analyses, each arm was further divided into two distinct regions: a zone one body length from the stimulus cage and the residual length of the arm, more remote from the cage.

Each test utilized one stimulus rat from each of four categories: a castrated, testosterone-treated male; a castrated male without hormone treatment; an ovariectomized, estradiol-treated female; and an ovariectomized female without hormone treatment. We refer to the stimulus rats relative to the experimental animal as opposite-sex (OS) or same-sex (SS); with (+) or without (-) hormone. Thus, the four stimulus options were: OS+, OS-, SS+, SS-. On the day of testing, stimulus rats were placed, in random order, into one of the four holding cages in the corners. Then, the experimental

animal was placed in the middle of the apparatus, marking the beginning of the 10-minute trial. Between tests, in addition to rearranging the stimulus animals' placement, we periodically replaced stimulus rats to minimize possible consequences of stimulus rat fatigue due to prolonged housing in the holding cages.

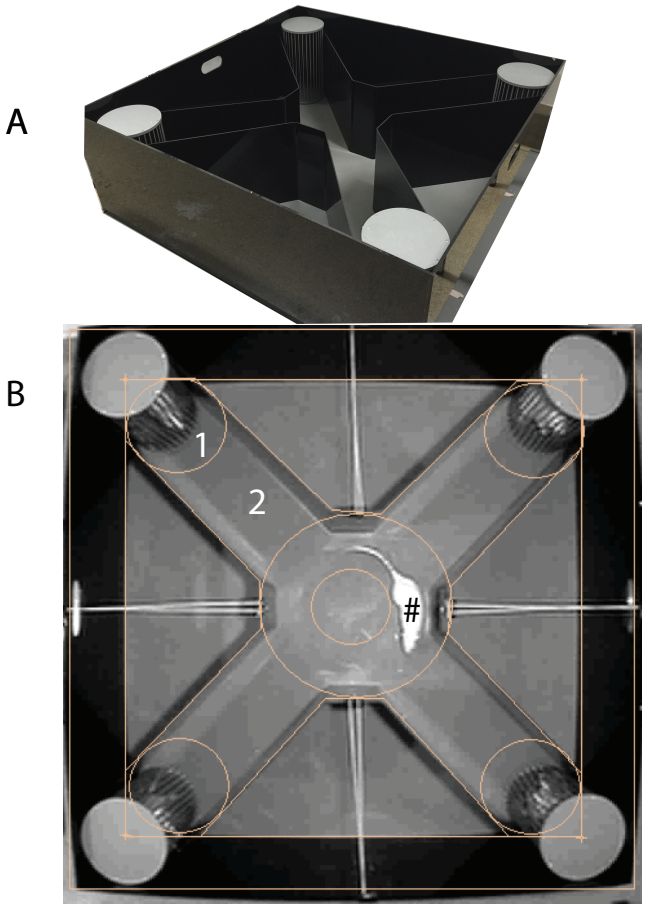



Figure 4.1. The FourPlex Apparatus

The FourPlex apparatus is shown viewed from one corner (A) and in a bird's eye view (B). Four opaque Plexiglas inserts were fabricated to fit inside of a square apparatus (100 cm x 100 cm), resulting in an X-shaped testing arena. As seen in B, each of the four arms emanated from a common central area (outer circle labeled "3") that projected via four arms to a holding cage in each corner. For the purposes of behavioral analyses, each arm was further divided into two distinct regions: a zone one body length from the walls of the stimulus cage (1; the "near" region), and the remaining length of the arm (2; the "remote" region). At the onset of the test, the experimental rat (indicated by #) was placed into the innermost center circle, labeled "4."

***Behavioral Analysis***

Animal tracking and behavior scoring. ANY-maze (Stoelting Co.) was used to simultaneously video record and track behaviors during the test. All scoring was done blind to the stimulus or experimental rats' status, and codes were broken only after scoring was complete. We used the software to analyze measures of duration or frequency relative to the animal's position within the apparatus, namely: the time spent in each whole arm ("Time in Whole Arm"); time spent in proximity to the stimulus rats' cage ("Time Near Stimulus Cage" = within one body length); and "Time in Remote Arm," calculated by subtracting the Time Near Stimulus Cage from the Time in Whole Arm. The number of times each animal entered (80% of body volume) an arm was automatically determined by the software. Then, the video recordings of the tests were manually scored for the following additional behaviors: "Number of Stimulus Explore Events" was the number of times an animal spent investigating the stimulus chamber by sniffing and exploring. The number of times the animals engaged in nose-to-nose contact, or "Number of Nose Touch Events," was also scored. These investigator-scored behaviors, chosen as the most salient aspect of social investigation, have been used before in a previous study on sociality (Reilly et al., 2015). The relative contribution each stimulus had on the cumulative expression of each behavior can be assessed qualitatively by graphing the means of each measure as a percentage of the whole (Figure 4.2).

During initial analysis, we established that the vast majority of the experimental animals had visited every stimulus option by the end of the first two minutes. When this happened, rats were able to make an 'Informed Choice' about all of their stimulus options. An example of a rat visiting each of the four stimulus animals is shown in the Supplemental video. Therefore, our assessment and presentation of social behavior in this study comprises the behavior of the animals during the latter eight-minute period of time

during which the experimental animals' behavior reflects this informed choice. Any animals that did not visit all four stimulus options during the two-minute habituation period were removed from all subsequent analyses. This resulted in two rats being excluded: one vehicle female, and one vehicle male.

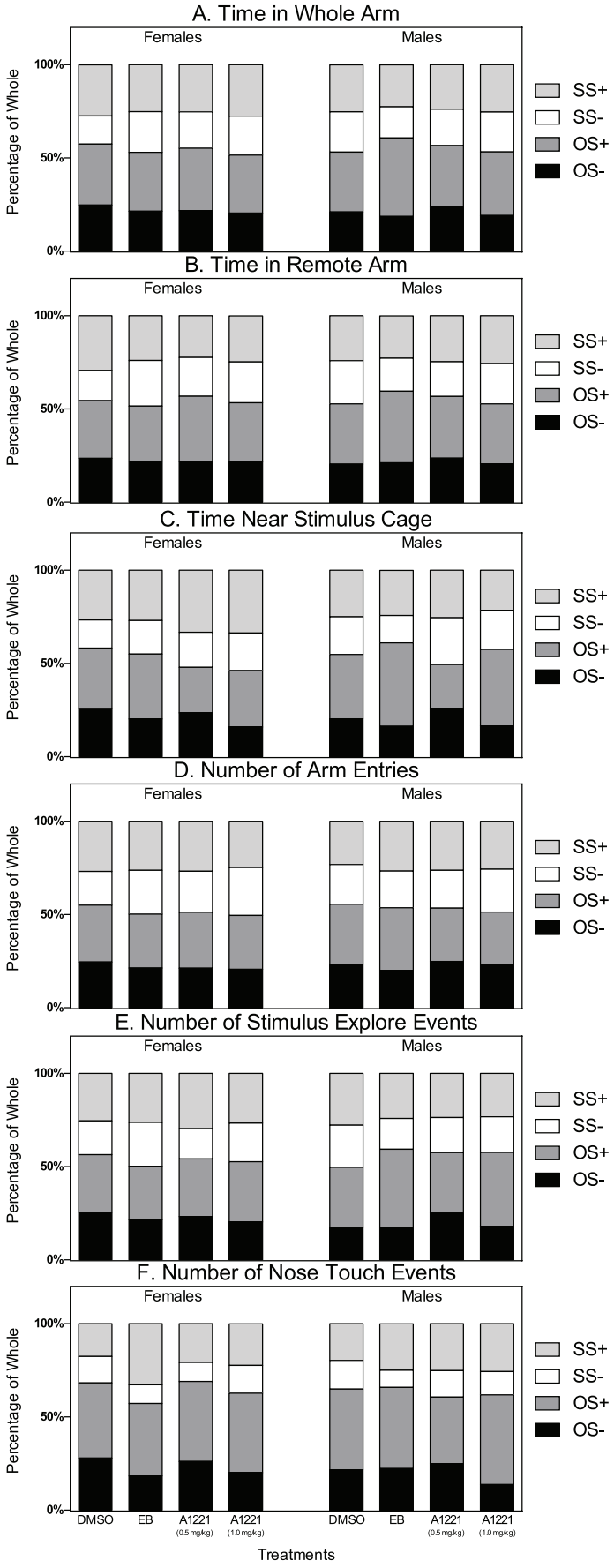



Figure 4.2. Qualitative Expression of Choice

The contribution each stimulus choice had on the behavioral output is shown for the six variables measured. Data are shown separately for each of the four treatment groups in females and males. Bars are always set at 100%, and the percentage of time each behavior is displayed toward each of the stimulus categories is shown. This enables visualization that most behaviors are predominantly exhibited towards the opposite-sex hormone-treated rat, which is the most socially salient, and validates that the FourPlex enables animals to make this distinction. Terminology here and throughout the manuscript is OS-: opposite-sex castrated rat (no hormone); OS+: opposite-sex castrated rat given hormone (estradiol in females, testosterone in males); SS-: same-sex castrated rat, no hormone; and SS+: same-sex castrated rat, given hormone.

## Statistical Analysis

### *Outliers*

A generalized extreme studentized deviate (ESD) test was used to detect outliers, limited to a maximum of two per group per endpoint. Any individuals repeatedly detected as outliers across multiple endpoints were removed from analyses; this was the case for two vehicle animals (one per sex) and one EB female. Initial statistical analyses were used to identify any potential cohort or litter effects within groups; none were found.

### *Statistical tests*

Because of the sexual dimorphism in social and sociosexual behavior, analyses were done separately for each sex. First, each dataset was inspected for homogeneity of variance and normality to determine if it met criteria for parametric analysis by ANOVA. Those endpoints that met criteria were analyzed this way using R (version 3.3.2), and effect sizes were calculated using the lsr package, and reported as partial eta-squared ($\eta p^2$) values in the tables. When data did not satisfy the assumptions for parametric statistics, the non-parametric Kruskal-Walis (KW) test was run using JMP (version 12), with effect sizes reported as epsilon-squared ($\varepsilon^2$) values. The Tukey HSD and Steel-Dwass all-pairs tests were used as post-hoc tests for the ANOVA and KW, respectively. In order to determine whether there were sex differences within treatment groups, a Student's t-test was used, and effect sizes were determined using Cohen's d values with the online effect size calculator (http://www.uccs.edu/~lbecker/). In the tables, those effects that were significant at $p < 0.05$ and/or had LARGE effect sizes are indicated with bold text.

### *Multivariate analyses*

Principle Components Analysis (PCA) was employed to analyze the entire behavioral ethogram within each sex (Scarpino, Gillette, & Crews, 2014; Gillette et al., 2014). The full list of behaviors in the ethogram is shown in Supplemental Figure 4.1. Rotation was applied such that the axis would capture the maximal amount of variance. Three principle components were identified as contributing to the majority of the variance (53%) in both sexes (Supplemental Figure 4.1). Then, linear discriminate analysis (LDA) was used to conduct systematic pairwise comparisons of each component for all animals, as well as within each sex, to establish how the principle components clustered (Supplemental Table 4.1). Finally, functional landscape analysis was conducted for sex differences on two behavioral outcomes, one representing an appetitive behavior, the other a consummatory behavior. This allows visual graphic representation and quantitative analysis of phenotypic traits first as absolute measures towards the four stimuli, as well as sex differences for each treatment group (Scarpino, Gillette, & Crews, 2014).

## Results

### *Overview of Behaviors in the FourPlex Test*

For each sex, data were initially analyzed for effects of treatment on total time and numbers of events during the 8-minute "informed choice" part of the test, irrespective of which stimulus rat to which they were directed (Table 4.2). There were no significant effects of treatment in females or males. We also determined sex differences. The number of nose touch events in the EB and A1221 (0.5) groups was significantly sexually dimorphic (male > female) and had LARGE Cohen's d effect sizes. The time

near the stimulus cage had a LARGE effect size for the sex difference in vehicle rats (female > male) although it was not statistically significantly different.

A. Female Treatment Effects

| Measure | Treatment | Mean (s) | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|
| Time in Whole Arm (sec) | Vehicle | 286 | ± 23 | Kruskal-Wallis | $X^2_{(3)} = 3.01$ | 0.39 | 0.08 |
| | EB | 297 | ± 14 | | | | |
| | A1221 (0.5) | 330 | ± 10 | | | | |
| | A1221 (1.0) | 301 | ± 19 | | | | |
| Time in Remote Arm (sec) | Vehicle | 160 | ± 21 | Kruskal-Wallis | $X^2_{(3)} = 5.92$ | 0.12 | 0.16 |
| | EB | 198 | ± 13 | | | | |
| | A1221 (0.5) | 225 | ± 13 | | | | |
| | A1221 (1.0) | 188 | ± 14 | | | | |
| Time Near Stimulus Cage (sec) | Vehicle | 126 | ± 18 | ANOVA | $F_{(3,34)} = 0.59$ | 0.63 | 0.05 |
| | EB | 99 | ± 16 | | | | |
| | A1221 (0.5) | 105 | ± 14 | | | | |
| | A1221 (1.0) | 114 | ± 11 | | | | |
| Number of Arm Entries | Vehicle | 96 | ± 6 | ANOVA | $F_{(3,34)} = 0.66$ | 0.58 | 0.06 |
| | EB | 95 | ± 6 | | | | |
| | A1221 (0.5) | 102 | ± 4 | | | | |
| | A1221 (1.0) | 104 | ± 7 | | | | |
| Number of Stimulus Explore Events | Vehicle | 137 | ± 26 | ANOVA | $F_{(3,34)} = 0.25$ | 0.86 | 0.02 |
| | EB | 137 | ± 21 | | | | |
| | A1221 (0.5) | 145 | ± 10 | | | | |
| | A1221 (1.0) | 156 | ± 15 | | | | |
| Number of Nose Touch Events | Vehicle | 24 | ± 5 | ANOVA | $F_{(3,34)} = 2.05$ | 0.13 | 0.16 |
| | EB | 21 | ± 5 | | | | |
| | A1221 (0.5) | 23 | ± 4 | | | | |
| | A1221 (1.0) | 33 | ± 3 | | | | |

(Table 4.2 continued on next page)

B. Male Treatment Effects

| Measure | Treatment | Mean (s) | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|
| Time in Whole Arm (sec) | Vehicle | 289 | ± 26 | Kruskal-Wallis | | $X^2_{(3)} = 7.50$ | 0.06 |
| | EB | 299 | ± 14 | | | | |
| | A1221 (0.5) | 349 | ± 16 | | | | |
| | A1221 (1.0) | 300 | ± 23 | | | | |
| Time in Remote Arm (sec) | Vehicle | 200 | ± 19 | Kruskal-Wallis | | $X^2_{(3)} = 0.68$ | 0.87 |
| | EB | 202 | ± 23 | | | | |
| | A1221 (0.5) | 241 | ± 23 | | | | |
| | A1221 (1.0) | 192 | ± 20 | | | | |
| Time Near Stimulus Cage (sec) | Vehicle | 89 | ± 12 | ANOVA | | $F_{(3,34)} = 0.62$ | 0.61 |
| | EB | 97 | ± 13 | | | | |
| | A1221 (0.5) | 108 | ± 14 | | | | |
| | A1221 (1.0) | 108 | ± 6 | | | | |
| Number of Arm Entries | Vehicle | 85 | ± 9 | Kruskal-Wallis | | $X^2_{(3)} = 7.37$ | 0.06 |
| | EB | 83 | ± 4 | | | | |
| | A1221 (0.5) | 92 | ± 8 | | | | |
| | A1221 (1.0) | 102 | ± 5 | | | | |
| Number of Stimulus Explore Events | Vehicle | 129 | ± 18 | Kruskal-Wallis | | $X^2_{(3)} = 3.58$ | 0.31 |
| | EB | 151 | ± 9 | | | | |
| | A1221 (0.5) | 179 | ± 19 | | | | |
| | A1221 (1.0) | 162 | ± 9 | | | | |
| Number of Nose Touch Events | Vehicle | 26 | ± 3 | ANOVA | | $F_{(3,34)} = 1.51$ | 0.23 |
| | EB | 32 | ± 3 | | | | |
| | A1221 (0.5) | 38 | ± 5 | | | | |
| | A1221 (1.0) | 35 | ± 6 | | | | |

(Table 4.2 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Measure | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| Time in Whole Arm (sec) | Vehicle | 0.94 | -0.04 | SMALL | |
| | EB | 0.89 | -0.06 | SMALL | |
| | A1221 (0.5) | 0.34 | -0.44 | SMALL | |
| | A1221 (1.0) | 0.95 | 0.03 | SMALL | |
| Time in Remote Arm (sec) | Vehicle | 0.30 | -0.52 | MEDIUM | |
| | EB | 0.91 | 0.05 | SMALL | |
| | A1221 (0.5) | 0.48 | 0.32 | SMALL | |
| | A1221 (1.0) | 0.62 | -0.23 | SMALL | |
| Time Near Stimulus Cage (sec) | Vehicle | 0.10 | **0.85** | **LARGE** | F > M |
| | EB | 0.94 | 0.04 | SMALL | |
| | A1221 (0.5) | 0.89 | -0.06 | SMALL | |
| | A1221 (1.0) | 0.66 | 0.20 | SMALL | |
| Number of Arm Entries | Vehicle | 0.35 | 0.48 | SMALL | |
| | EB | 0.12 | 0.74 | MEDIUM | |
| | A1221 (0.5) | 0.26 | 0.52 | MEDIUM | |
| | A1221 (1.0) | 0.75 | 0.15 | SMALL | |
| Number of Stimulus Explore Events | Vehicle | 0.79 | 0.13 | SMALL | |
| | EB | 0.54 | -0.28 | SMALL | |
| | A1221 (0.5) | 0.12 | -0.72 | MEDIUM | |
| | A1221 (1.0) | 0.73 | -0.16 | SMALL | |
| Number of Nose Touch Events | Vehicle | 0.72 | -0.17 | SMALL | |
| | EB | **0.05** | **-0.96** | **LARGE** | M > F |
| | A1221 (0.5) | **0.02** | **-1.17** | **LARGE** | M > F |
| | A1221 (1.0) | 0.80 | -0.12 | SMALL | |

Table 4.2. Summary of Behaviors

A summary of all behaviors is shown for Female (A) and Male (B) rats. Data are shown as mean + SEM, statistical tests, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). No significant treatment effects were found for either sex. (C) The statistics for within-treatment sex differences and Cohen's d effect sizes for the sex differences are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8), with directionality indicated (M=male, F=female). Where there are significant differences or LARGE effect sizes, data are shown in bold text.

**Social Investigation of Stimulus Animals**

Each of the behaviors was subsequently analyzed for effects of treatment and sex, with the 4 stimulus rats as variables. The following analyses were conducted on the 8-minute "informed choice" period.

***Time in Whole Arm (Table 4.3; Figure 4.2A)***

Regardless of sex or treatment, experimental rats spent the most time in the arm leading up to the OS+ stimulus animal. However, there were no main effects of treatment. There was one significant sex difference found for the EB groups towards the OS+ stimulus rat (male > female), with a LARGE Cohen's d effect size.

| A. Female Treatment Effects | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Stimulus | Treatment | Mean (s) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
| OS- | Vehicle | 77 | ± | 7 | ANOVA | $F_{(3,33)} = 1.52$ | 0.23 | 0.12 |
| | EB | 63 | ± | 4 | | | | |
| | A1221 (0.5) | 72 | ± | 4 | | | | |
| | A1221 (1.0) | 63 | ± | 7 | | | | |
| OS+ | Vehicle | 102 | ± | 12 | Kruskal-Wallis | $X^2_{(3)} = 2.37$ | 0.50 | 0.07 |
| | EB | 92 | ± | 5 | | | | |
| | A1221 (0.5) | 111 | ± | 11 | | | | |
| | A1221 (1.0) | 96 | ± | 10 | | | | |
| SS- | Vehicle | 47 | ± | 6 | ANOVA | $F_{(3,33)} = 2.27$ | 0.10 | 0.18 |
| | EB | 65 | ± | 7 | | | | |
| | A1221 (0.5) | 64 | ± | 5 | | | | |
| | A1221 (1.0) | 65 | ± | 4 | | | | |
| SS+ | Vehicle | 86 | ± | 10 | ANOVA | $F_{(3,32)} = 0.67$ | 0.58 | 0.06 |
| | EB | 75 | ± | 7 | | | | |
| | A1221 (0.5) | 83 | ± | 6 | | | | |
| | A1221 (1.0) | 85 | ± | 4 | | | | |

(Table 4.3 continued on next page)

B. Male Treatment Effects

| Stimulus | Treatment | Mean (s) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 62 | ± | 8 | ANOVA | $F_{(3,35)} = 2.76$ | 0.06 | 0.20 |
| | EB | 58 | ± | 6 | | | | |
| | A1221 (0.5) | 82 | ± | 6 | | | | |
| | A1221 (1.0) | 61 | ± | 7 | | | | |
| OS+ | Vehicle | 96 | ± | 9 | ANOVA | $F_{(3,35)} = 2.14$ | 0.11 | 0.16 |
| | EB | 132 | ± | 9 | | | | |
| | A1221 (0.5) | 115 | ± | 13 | | | | |
| | A1221 (1.0) | 109 | ± | 8 | | | | |
| SS- | Vehicle | 64 | ± | 9 | ANOVA | $F_{(3,36)} = 0.99$ | 0.41 | 0.08 |
| | EB | 52 | ± | 7 | | | | |
| | A1221 (0.5) | 68 | ± | 8 | | | | |
| | A1221 (1.0) | 68 | ± | 7 | | | | |
| SS+ | Vehicle | 75 | ± | 8 | ANOVA | $F_{(3,33)} = 0.61$ | 0.61 | 0.05 |
| | EB | 70 | ± | 8 | | | | |
| | A1221 (0.5) | 83 | ± | 8 | | | | |
| | A1221 (1.0) | 81 | ± | 5 | | | | |

(Table 4.3 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Stimulus | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| OS- | Vehicle | 0.66 | 0.76 | MEDIUM | |
| | EB | 0.52 | 0.40 | SMALL | |
| | A1221 (0.5) | 0.13 | -0.72 | MEDIUM | |
| | A1221 (1.0) | 0.80 | 0.12 | SMALL | |
| OS+ | Vehicle | 0.67 | 0.21 | SMALL | |
| | EB | **0.002** | **-1.58** | **LARGE** | M > F |
| | A1221 (0.5) | 0.80 | -0.12 | SMALL | |
| | A1221 (1.0) | 0.37 | -0.43 | SMALL | |
| SS- | Vehicle | 0.81 | -0.78 | MEDIUM | |
| | EB | 0.14 | 0.61 | MEDIUM | |
| | A1221 (0.5) | 0.69 | -0.18 | SMALL | |
| | A1221 (1.0) | 0.69 | -0.18 | SMALL | |
| SS+ | Vehicle | 0.39 | 0.44 | SMALL | |
| | EB | 0.77 | 0.21 | SMALL | |
| | A1221 (0.5) | 0.97 | 0.01 | SMALL | |
| | A1221 (1.0) | 0.51 | 0.31 | SMALL | |

Table 4.3. Time in Whole Arm (seconds)

Time spent in the whole arm (seconds) is shown for Females (A) and Males (B). Data here, and in subsequent Tables 4-7, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There were no differences due to treatment. The one significant sex difference is indicated. M=male, F=female.

***Time in Remote Arm (Table 4.4; Figure 4.2B)***

Rats spent the most time in the OS+ stimulus animals' arm. In females, there was a main effect of treatment within the SS- arm, with post-hoc analysis indicating that the EB, A1221 (0.5) and A1221 (1.0) females spent significantly more time there than did the vehicle females. Regarding sex differences, a significant difference was observed in the EB group toward the OS+ stimulus animal (male > female), with a LARGE effect size. The other significant sex difference was seen in the vehicle group toward the SS- stimulus animal (male > female), again with a LARGE effect size.

A. Female Treatment Effects

| Stimulus | Treatment | Mean (s) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 33 | ± | 9 | Kruskal-Wallis | $X^2_{(3)} = 2.06$ | 0.56 | 0.06 |
| | EB | 42 | ± | 4 | | | | |
| | A1221 (0.5) | 46 | ± | 5 | | | | |
| | A1221 (1.0) | 46 | ± | 7 | | | | |
| OS+ | Vehicle | 63 | ± | 15 | Kruskal-Wallis | $X^2_{(3)} = 5.67$ | 0.13 | 0.16 |
| | EB | 57 | ± | 7 | | | | |
| | A1221 (0.5) | 89 | ± | 12 | | | | |
| | A1221 (1.0) | 61 | ± | 9 | | | | |
| SS- | Vehicle | 21 | ± | 7 | Kruskal-Wallis | $X^2_{(3)} = 11.67$ | **0.01** | **0.32** |
| | EB | 52 | ± | 7 | | | | |
| | A1221 (0.5) | 43 | ± | 5 | | | | |
| | A1221 (1.0) | 42 | ± | 3 | | | | |
| SS+ | Vehicle | 43 | ± | 10 | Kruskal-Wallis | $X^2_{(3)} = 0.0597$ | 1.00 | 0.00 |
| | EB | 46 | ± | 5 | | | | |
| | A1221 (0.5) | 47 | ± | 5 | | | | |
| | A1221 (1) | 38 | ± | 10 | | | | |

(Table 4.4 continued on next page)

B. Male Treatment Effects

| Stimulus | Treatment | Mean (s) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 45 | ± | 7 | Kruskal-Wallis | $X^2_{(3)} = 1.83$ | 0.61 | 0.05 |
| | EB | 35 | ± | 10 | | | | |
| | A1221 (0.5) | 54 | ± | 7 | | | | |
| | A1221 (1.0) | 43 | ± | 5 | | | | |
| OS+ | Vehicle | 62 | ± | 7 | Kruskal-Wallis | $X^2_{(3)} = 4.08$ | 0.25 | 0.11 |
| | EB | 93 | ± | 12 | | | | |
| | A1221 (0.5) | 95 | ± | 18 | | | | |
| | A1221 (1.0) | 52 | ± | 15 | | | | |
| SS- | Vehicle | 45 | ± | 6 | ANOVA | $F_{(3,36)} = 0.89$ | 0.46 | 0.07 |
| | EB | 36 | ± | 4 | | | | |
| | A1221 (0.5) | 37 | ± | 5 | | | | |
| | A1221 (1.0) | 45 | ± | 4 | | | | |
| SS+ | Vehicle | 48 | ± | 9 | Kruskal-Wallis | $X^2_{(3)} = 2.26$ | 0.48 | 0.06 |
| | EB | 37 | ± | 10 | | | | |
| | A1221 (0.5) | 55 | ± | 7 | | | | |
| | A1221 (1) | 51 | ± | 11 | | | | |

(Table 4.4 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Stimulus | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| OS- | EB | 0.76 | -0.19 | SMALL | |
| | A1221 (0.5) | 0.80 | -0.12 | SMALL | |
| | A1221 (1.0) | 0.82 | -0.11 | SMALL | |
| OS+ | Vehicle | 0.21 | -0.38 | SMALL | |
| | EB | **0.05** | **-1.11** | **LARGE** | M > F |
| | A1221 (0.5) | 0.64 | 0.25 | SMALL | |
| | A1221 (1.0) | 0.56 | -0.28 | SMALL | |
| SS- | Vehicle | **0.02** | **-1.23** | **LARGE** | M > F |
| | EB | 0.09 | 0.60 | MEDIUM | |
| | A1221 (0.5) | 0.39 | 0.40 | SMALL | |
| | A1221 (1.0) | 0.51 | -0.30 | SMALL | |
| SS+ | Vehicle | 0.63 | 0.17 | SMALL | |
| | EB | 0.97 | -0.12 | SMALL | |
| | A1221 (0.5) | 0.72 | -0.17 | SMALL | |
| | A1221 (1) | 0.13 | -0.78 | MEDIUM | |

Table 4.4. Time in Remote Arm (seconds)

Time spent in the remote arm (seconds) is shown with significant ($p < 0.05$) and/or LARGE effect sizes indicated. See Table 4.2 for explanations of statistics and data presentation.

***Time Near Stimulus Cage (Table 4.5; Figure 4.2C)***

There were no significant treatment effects for this measure, although there was a trend toward significance in both the males ($p = 0.06$) and the females ($p = 0.10$) for the OS- arm. Regarding sex differences, for the OS- arm, there was a LARGE effect size

(female > male) although this did not attain significance. Two significant sex differences were seen for the SS+ arm for vehicle and A1221 (1.0) groups, associated with a MEDIUM and LARGE Cohen's d effect size, respectively (female > male for both).

| A. Female Treatment Effects | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Stimulus | Treatment | Mean (s) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
| OS- | Vehicle | 35 | ± | 7 | ANOVA | $F_{(3,33)} = 2.25$ | 0.10 | 0.17 |
| | EB | 21 | ± | 4 | | | | |
| | A1221 (0.5) | 26 | ± | 4 | | | | |
| | A1221 (1.0) | 18 | ± | 4 | | | | |
| OS+ | Vehicle | 44 | ± | 8 | ANOVA | $F_{(3,32)} = 1.05$ | 0.38 | 0.10 |
| | EB | 35 | ± | 7 | | | | |
| | A1221 (0.5) | 27 | ± | 4 | | | | |
| | A1221 (1.0) | 35 | ± | 6 | | | | |
| SS- | Vehicle | 20 | ± | 4 | ANOVA | $F_{(3,32)} = 0.13$ | 0.94 | 0.01 |
| | EB | 20 | ± | 3 | | | | |
| | A1221 (0.5) | 21 | ± | 5 | | | | |
| | A1221 (1.0) | 23 | ± | 4 | | | | |
| SS+ | Vehicle | 36 | ± | 9 | ANOVA | $F_{(3,33)} = 0.70$ | 0.56 | 0.06 |
| | EB | 27 | ± | 7 | | | | |
| | A1221 (0.5) | 37 | ± | 6 | | | | |
| | A1221 (1.0) | 39 | ± | 5 | | | | |

(Table 4.5 continued on next page)

B. Male Treatment Effects

| Stimulus | Treatment | Mean (s) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 20 | ± | 4 | ANOVA | $F_{(3,34)} = 2.69$ | 0.06 | 0.20 |
| | EB | 18 | ± | 4 | | | | |
| | A1221 (0.5) | 32 | ± | 5 | | | | |
| | A1221 (1.0) | 18 | ± | 3 | | | | |
| OS+ | Vehicle | 33 | ± | 5 | ANOVA | $F_{(3,31)} = 2.41$ | 0.09 | 0.19 |
| | EB | 48 | ± | 4 | | | | |
| | A1221 (0.5) | 29 | ± | 5 | | | | |
| | A1221 (1.0) | 45 | ± | 7 | | | | |
| SS- | Vehicle | 19 | ± | 5 | Kruskal-Wallis | $X^2_{(3)} = 3.28$ | 0.35 | 0.09 |
| | EB | 16 | ± | 3 | | | | |
| | A1221 (0.5) | 31 | ± | 8 | | | | |
| | A1221 (1.0) | 23 | ± | 4 | | | | |
| SS+ | Vehicle | 24 | ± | 5 | ANOVA | $F_{(3,32)} = 0.41$ | 0.74 | 0.04 |
| | EB | 26 | ± | 6 | | | | |
| | A1221 (0.5) | 31 | ± | 7 | | | | |
| | A1221 (1.0) | 24 | ± | 3 | | | | |

(Table 4.5 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Stimulus | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| OS- | Vehicle | 0.10 | **0.90** | **LARGE** | F > M |
| | EB | 0.53 | 0.43 | MEDIUM | |
| | A1221 (0.5) | 0.38 | -0.41 | SMALL | |
| | A1221 (1.0) | 0.95 | 0.03 | SMALL | |
| OS+ | Vehicle | 0.35 | 0.60 | MEDIUM | |
| | EB | 0.16 | -0.48 | SMALL | |
| | A1221 (0.5) | 0.78 | -0.15 | SMALL | |
| | A1221 (1.0) | 0.29 | -0.49 | SMALL | |
| SS- | Vehicle | 0.71 | 0.07 | SMALL | |
| | EB | 0.42 | 0.47 | SMALL | |
| | A1221 (0.5) | 0.27 | -0.51 | MEDIUM | |
| | A1221 (1.0) | 0.97 | -0.02 | SMALL | |
| SS+ | Vehicle | **0.04** | **0.66** | **MEDIUM** | F > M |
| | EB | 0.87 | 0.22 | SMALL | |
| | A1221 (0.5) | 0.53 | 0.29 | SMALL | |
| | A1221 (1.0) | **0.02** | **1.24** | **LARGE** | F > M |

Table 4.5. Time Near Stimulus Cage (seconds)

Time spent near (one body length) each stimulus rat's holding cage is shown with significant ($p < 0.05$) and/or LARGE effect sizes indicated. See Table 4.2 for explanations of statistics and data presentation.

***Number of Arm Entries (Table 4.6; Figure 4.2D)***

All treatment groups entered the arm containing the OS+ stimulus animal most often. A main effect of treatment was seen in females for the SS- arm, driven by a significantly higher number of entries for the A1221 (1.0) females entered the arm compared to females in the vehicle group. There were no main effects of treatment in males. There was one instance of a significant sex difference for EB rats with respect to the SS- stimulus (female > male); this difference was associated a LARGE effect size.

B. Male Treatment Effects

| Stimulus | Treatment | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 21 | ± | 1 | Kruskal-Wallis | $X^2_{(3)} = 7.30$ | 0.06 | 0.20 |
| | EB | 17 | ± | 1 | | | | |
| | A1221 (0.5) | 23 | ± | 2 | | | | |
| | A1221 (1.0) | 24 | ± | 3 | | | | |
| OS+ | Vehicle | 29 | ± | 3 | Kruskal-Wallis | $X^2_{(3)} = 0.79$ | 0.85 | 0.02 |
| | EB | 29 | ± | 3 | | | | |
| | A1221 (0.5) | 26 | ± | 3 | | | | |
| | A1221 (1.0) | 28 | ± | 3 | | | | |
| SS- | Vehicle | 19 | ± | 3 | ANOVA | $X^2_{(3)} = 1.37$ | 0.27 | 0.11 |
| | EB | 17 | ± | 2 | | | | |
| | A1221 (0.5) | 19 | ± | 3 | | | | |
| | A1221 (1.0) | 23 | ± | 2 | | | | |
| SS+ | Vehicle | 21 | ± | 1 | Kruskal-Wallis | $X^2_{(3)} = 3.09$ | 0.38 | 0.08 |
| | EB | 23 | ± | 2 | | | | |
| | A1221 (0.5) | 24 | ± | 3 | | | | |
| | A1221 (1.0) | 26 | ± | 2 | | | | |

A. Female Treatment Effects

| Stimulus | Treatment | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 24 | ± | 2 | ANOVA | $F_{(3,34)} = 0.75$ | 0.53 | 0.06 |
| | EB | 20 | ± | 1 | | | | |
| | A1221 (0.5) | 22 | ± | 1 | | | | |
| | A1221 (1.0) | 22 | ± | 2 | | | | |
| OS+ | Vehicle | 29 | ± | 4 | Kruskal-Wallis | $X^2_{(3)} = 1.04$ | 0.79 | 0.03 |
| | EB | 27 | ± | 1 | | | | |
| | A1221 (0.5) | 31 | ± | 3 | | | | |
| | A1221 (1.0) | 30 | ± | 4 | | | | |
| SS- | Vehicle | 17 | ± | 2 | ANOVA | $F_{(3,34)} = 5.12$ | **0.01** | **0.32** |
| | EB | 22 | ± | 2 | | | | |
| | A1221 (0.5) | 22 | ± | 1 | | | | |
| | A1221 (1.0) | 27 | ± | 2 | | | | |
| SS+ | Vehicle | 26 | ± | 3 | ANOVA | $F_{(3,34)} = 0.23$ | 0.87 | 0.02 |
| | EB | 25 | ± | 2 | | | | |
| | A1221 (0.5) | 27 | ± | 2 | | | | |
| | A1221 (1.0) | 26 | ± | 1 | | | | |

(Table 4.6 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Stimulus | Treatment | P-Value | Cohen's d | Effect size | Directionality |
| OS- | Vehicle | 0.78 | 0.56 | MEDIUM | |
| | EB | 0.16 | 0.76 | MEDIUM | |
| | A1221 (0.5) | 0.62 | -0.23 | SMALL | |
| | A1221 (1.0) | 0.54 | -0.28 | SMALL | |
| OS+ | Vehicle | 0.54 | 0.02 | SMALL | |
| | EB | 0.69 | -0.18 | SMALL | |
| | A1221 (0.5) | 0.27 | 0.51 | MEDIUM | |
| | A1221 (1.0) | 0.74 | 0.15 | SMALL | |
| SS- | Vehicle | 0.61 | -0.25 | SMALL | |
| | EB | **0.05** | **0.81** | **LARGE** | F > M |
| | A1221 (0.5) | 0.21 | 0.58 | MEDIUM | |
| | A1221 (1.0) | 0.16 | 0.65 | MEDIUM | |
| SS+ | Vehicle | 0.17 | 0.71 | MEDIUM | |
| | EB | 0.65 | 0.28 | SMALL | |
| | A1221 (0.5) | 0.45 | 0.35 | SMALL | |
| | A1221 (1.0) | 0.93 | -0.04 | SMALL | |

Table 4.6. Number of Arm Entries

The mean number of entries into each of the arms is shown with significant ($p < 0.05$) and/or LARGE effect sizes indicated. See Table 4.2 for explanations of statistics and data presentation.

***Number of Stimulus Explore Events (Table 4.7; Figure 4.2E)***

For all treatments and both sexes, the number of times the experimental animal explored the stimulus cage was highest toward the OS+ animal. No effects of treatment were seen in females. A main effect of treatment was observed in the males towards the

OS- stimulus rats, with post-hoc analysis showing A1221 (0.5) > vehicle and EB. Several main effects of sex were observed. Compared to EB females, EB males explored the OS+ stimulus significantly more; this difference was associated with a LARGE effect size. With respect to the SS- stimulus, a main effect of sex was found for the EB (female > male) and A1221 (0.5) (male > female). The Cohen's d effect sizes associated with these differences were both LARGE.

A. Female Treatment Effects

| Stimulus | Treatment | Mean (#) | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 36 | ± 9 | ANOVA | $F_{(3,34)} = 0.18$ | 0.91 | 0.02 |
| | EB | 34 | ± 6 | | | | |
| | A1221 (0.5) | 35 | ± 4 | | | | |
| | A1221 (1.0) | 33 | ± 6 | | | | |
| OS+ | Vehicle | 44 | ± 8 | Kruskal-Wallis | $X^2_{(3)} = 1.37$ | 0.71 | 0.04 |
| | EB | 46 | ± 2 | | | | |
| | A1221 (0.5) | 47 | ± 5 | | | | |
| | A1221 (1.0) | 52 | ± 6 | | | | |
| SS- | Vehicle | 26 | ± 6 | Kruskal-Wallis | $X^2_{(3)} = 5.15$ | 0.16 | 0.15 |
| | EB | 37 | ± 3 | | | | |
| | A1221 (0.5) | 25 | ± 2 | | | | |
| | A1221 (1.0) | 33 | ± 5 | | | | |
| SS+ | Vehicle | 36 | ± 8 | ANOVA | $F_{(3,32)} = 0.39$ | 0.76 | 0.04 |
| | EB | 42 | ± 8 | | | | |
| | A1221 (0.5) | 45 | ± 7 | | | | |
| | A1221 (1.0) | 43 | ± 5 | | | | |

(Table 4.7 continued on next page)

B. Male Treatment Effects

| Stimulus | Treatment | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 24 | ± | 5 | ANOVA | $F_{(3,33)} = 4.13$ | **0.01** | **0.28** |
| | EB | 26 | ± | 4 | | | | |
| | A1221 (0.5) | 47 | ± | 6 | | | | |
| | A1221 (1.0) | 31 | ± | 5 | | | | |
| OS+ | Vehicle | 45 | ± | 7 | ANOVA | $F_{(3,35)} = 1.41$ | 0.26 | 0.11 |
| | EB | 65 | ± | 5 | | | | |
| | A1221 (0.5) | 60 | ± | 11 | | | | |
| | A1221 (1.0) | 69 | ± | 10 | | | | |
| SS- | Vehicle | 32 | ± | 3 | ANOVA | $F_{(3,34)} = 1.83$ | 0.16 | 0.14 |
| | EB | 25 | ± | 3 | | | | |
| | A1221 (0.5) | 35 | ± | 4 | | | | |
| | A1221 (1.0) | 33 | ± | 2 | | | | |
| SS+ | Vehicle | 39 | ± | 5 | ANOVA | $F_{(3,34)} = 0.43$ | 0.73 | 0.04 |
| | EB | 37 | ± | 4 | | | | |
| | A1221 (0.5) | 44 | ± | 5 | | | | |
| | A1221 (1.0) | 40 | ± | 4 | | | | |

(Table 4.7 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Stimulus | Treatment | P-Value | Cohen's d | Effect size | Directionality |
| OS- | Vehicle | 0.80 | 0.58 | MEDIUM | |
| | EB | 0.55 | 0.50 | MEDIUM | |
| | A1221 (0.5) | 0.15 | -0.68 | MEDIUM | |
| | A1221 (1.0) | 0.83 | 0.10 | SMALL | |
| OS+ | Vehicle | 0.83 | -0.06 | SMALL | |
| | EB | **0.01** | **-1.61** | **LARGE** | M > F |
| | A1221 (0.5) | 0.29 | -0.52 | MEDIUM | |
| | A1221 (1.0) | 0.17 | -0.63 | MEDIUM | |
| SS- | Vehicle | 0.69 | -0.42 | MEDIUM | |
| | EB | **0.03** | **1.31** | **LARGE** | F > M |
| | A1221 (0.5) | **0.03** | **-1.12** | **LARGE** | M > F |
| | A1221 (1.0) | 0.97 | 0.02 | SMALL | |
| SS+ | Vehicle | 0.84 | -0.13 | SMALL | |
| | EB | 0.95 | 0.23 | SMALL | |
| | A1221 (0.5) | 0.87 | 0.07 | SMALL | |
| | A1221 (1.0) | 0.67 | 0.20 | SMALL | |

Table 4.7. Number of Stimulus Explore Events

The mean number of stimulus explore events is shown with significant ($p < 0.05$) and/or LARGE effect sizes indicated. See Table 4.2 for explanations of statistics and data presentation.

***Number of Nose Touch Events (Table 4.8; Figure 4.2F)***

The OS+ stimulus animals again elicited the greatest number of nose touches, and there were no main effects of treatment in either sex. There were several sex differences, all male > female, as indicated by p-values and/or LARGE effect sizes. Specifically, sex differences were found for the OS- stimulus [A1221 (0.5)]; for the OS+ stimulus (EB); and for both the SS- and the SS+ stimuli [A1221 (0.5)].

A. Female Treatment Effects

| Stimulus | Treatment | Mean (#) | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 6.8 | ± 2.1 | Kruskal-Wallis | $X^2_{(3)} = 1.42$ | 0.70 | 0.04 |
| | EB | 4.4 | ± 1.2 | | | | |
| | A1221 (0.5) | 6.3 | ± 1.2 | | | | |
| | A1221 (1.0) | 6.8 | ± 1.9 | | | | |
| OS+ | Vehicle | 9.8 | ± 2.4 | Kruskal-Wallis | $X^2_{(3)} = 4.08$ | 0.25 | 0.12 |
| | EB | 7.8 | ± 2.5 | | | | |
| | A1221 (0.5) | 10.3 | ± 2.4 | | | | |
| | A1221 (1.0) | 14.4 | ± 2.4 | | | | |
| SS- | Vehicle | 3.4 | ± 1.6 | Kruskal-Wallis | $X^2_{(3)} = 3.67$ | 0.30 | 0.11 |
| | EB | 1.9 | ± 0.8 | | | | |
| | A1221 (0.5) | 2.4 | ± 0.6 | | | | |
| | A1221 (1.0) | 5.0 | ± 1.3 | | | | |
| SS+ | Vehicle | 4.3 | ± 1.6 | Kruskal-Wallis | $X^2_{(3)} = 2.67$ | 0.45 | 0.08 |
| | EB | 7.9 | ± 2.3 | | | | |
| | A1221 (0.5) | 5.0 | ± 1.4 | | | | |
| | A1221 (1.0) | 7.6 | ± 1.2 | | | | |

B. Male Treatment Effects

| Stimulus | Treatment | Mean (#) | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|
| OS- | Vehicle | 5.8 | ± 2.2 | Kruskal-Wallis | $X^2_{(3)} = 4.43$ | 0.22 | 0.12 |
| | EB | 7.3 | ± 1.9 | | | | |
| | A1221 (0.5) | 10.0 | ± 1.6 | | | | |
| | A1221 (1.0) | 4.9 | ± 1.3 | | | | |
| OS+ | Vehicle | 11.6 | ± 3.2 | Kruskal-Wallis | $X^2_{(3)} = 1.90$ | 0.59 | 0.05 |
| | EB | 14.3 | ± 1.8 | | | | |
| | A1221 (0.5) | 14.3 | ± 2.5 | | | | |
| | A1221 (1.0) | 17.1 | ± 3.9 | | | | |
| SS- | Vehicle | 4.1 | ± 1.0 | ANOVA | $F_{(3,33)} = 1.30$ | 0.29 | 0.11 |
| | EB | 3.0 | ± 0.6 | | | | |
| | A1221 (0.5) | 5.7 | ± 1.0 | | | | |
| | A1221 (1.0) | 4.4 | ± 1.2 | | | | |
| SS+ | Vehicle | 5.3 | ± 1.1 | Kruskal-Wallis | $X^2_{(3)} = 3.73$ | 0.29 | 0.10 |
| | EB | 8.2 | ± 1.7 | | | | |
| | A1221 (0.5) | 10.1 | ± 2.1 | | | | |
| | A1221 (1.0) | 9.1 | ± 1.7 | | | | |

(Table 4.8 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Stimulus | Treatment | P-Value | Cohen's d | Effect size | Directionality |
| OS- | Vehicle | 0.41 | 0.15 | SMALL | |
| | EB | 0.23 | -0.55 | MEDIUM | |
| | A1221 (0.5) | 0.09 | **-0.81** | **LARGE** | M > F |
| | A1221 (1.0) | 0.43 | 0.38 | SMALL | |
| OS+ | Vehicle | 0.06 | -0.21 | SMALL | |
| | EB | **0.01** | **-0.64** | **MEDIUM** | M > F |
| | A1221 (0.5) | 0.26 | -0.55 | MEDIUM | |
| | A1221 (1.0) | 0.56 | -0.26 | SMALL | |
| SS- | Vehicle | 0.18 | -0.19 | SMALL | |
| | EB | 0.22 | -0.29 | SMALL | |
| | A1221 (0.5) | **0.01** | **-1.34** | **LARGE** | M > F |
| | A1221 (1.0) | 0.77 | 0.14 | SMALL | |
| SS+ | Vehicle | 0.09 | -0.26 | SMALL | |
| | EB | 0.92 | 0.03 | SMALL | |
| | A1221 (0.5) | 0.06 | **-0.90** | **LARGE** | M > F |
| | A1221 (1.0) | 0.48 | -0.33 | SMALL | |

Table 4.8. Number of Nose Touch Events

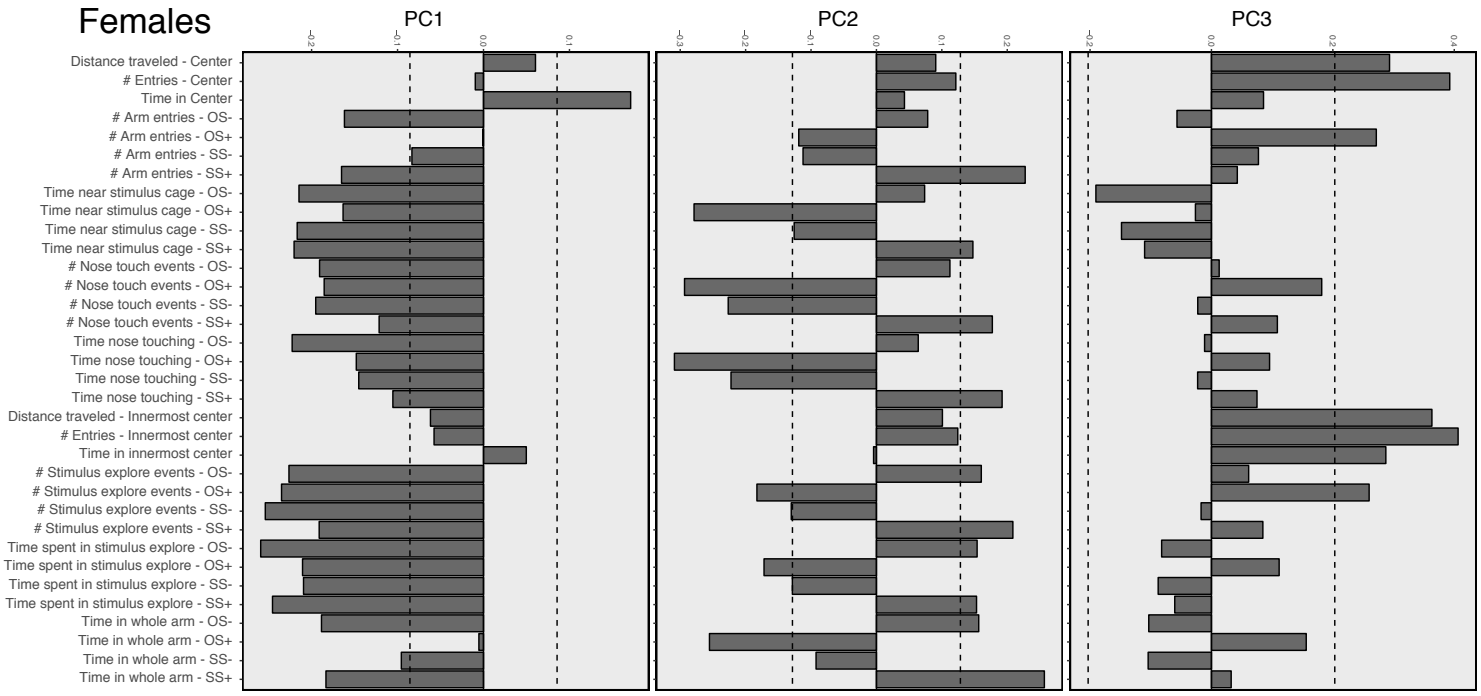
The mean number of nose touch events is shown with significant ($p < 0.05$) and/or LARGE effect sizes indicated. See Table 2 for explanations of statistics and data presentation.

## Multivariate Analyses of Behavior

### *Principle components analysis and linear discriminate analysis.*

In females, PCA determined that 53% of the variance could be accounted for within the first 3 principle components (Figure 4.3A). Linear discriminate analysis (Table 4.9) revealed that the EB and vehicle datasets differed from each other significantly within females. In males, again, PCA determined that 53% of the variance could be accounted for within the first 3 principle components (Figure 4.3B). There were no

effects of treatment found on the datasets within the males. When the sexes were considered together, female EB and male EB rats also differed (Table 4.9).




(Figure 4.3 continued on next page)

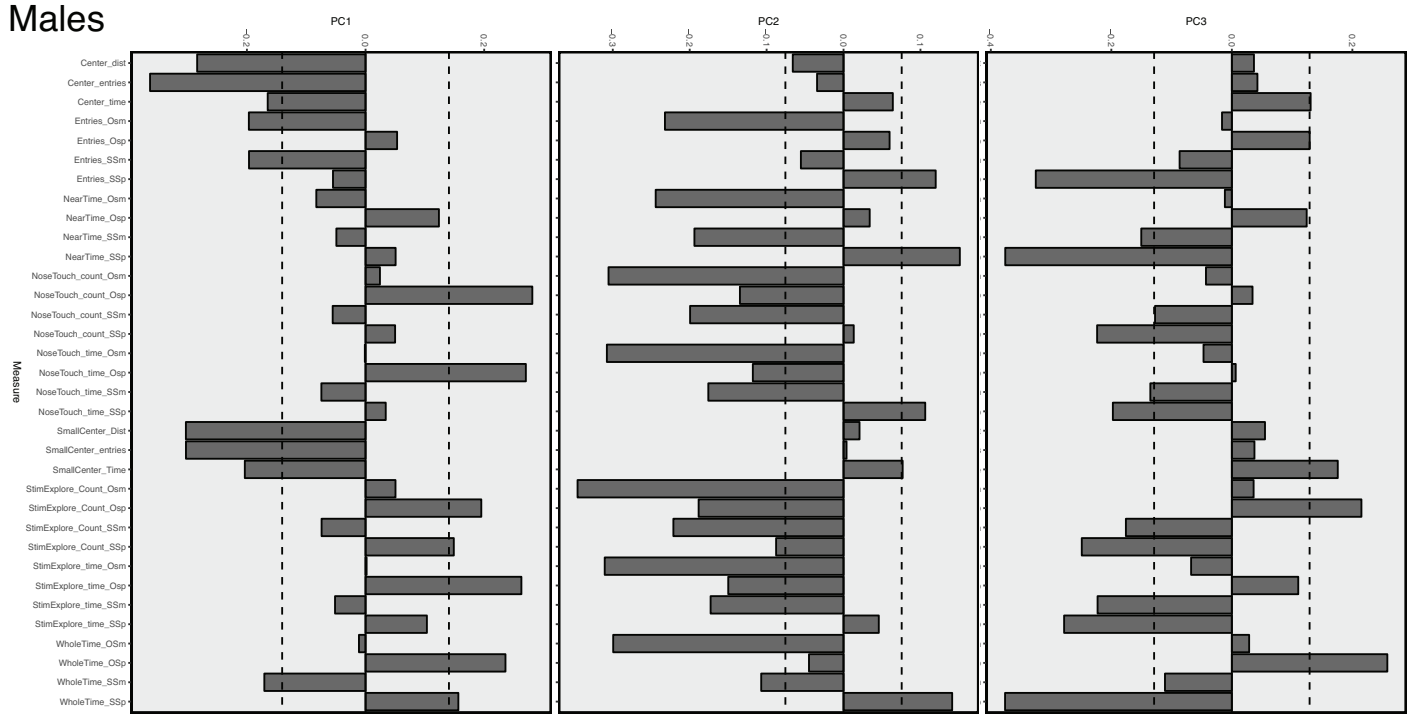



Figure 4.3. Principle Component Plots 1-3

Correlation plots derived from analysis of the first three principle components are shown for females (A) and males (B). These principle components, accounting for 53% of variance in both sexes, were derived from the analysis of the entire ethogram, with behaviors indicated on the y-axis. Along with those behaviors discussed elsewhere in the manuscript, behaviors relative to the center of the chamber are indicated with reference to the full center area (labeled "3" in Figure 4.1) or an innermost center (labeled "4" in Figure 4.1). For each measure listed on the vertical axis, the bar displays the magnitude and direction of correlation (loading) on each of the principle components 1-3, expressed as a correlation coefficient on the horizontal axes. Bars in the same direction indicate measures that correlate together. The dashed line indicates a threshold beyond which any measure can be stated to significantly influence each principle component, defined as one half the absolute value of the largest loading.

| A. All Animals | | | |
|---|---|---|---|
| Comparison | | Probability | Distance |
| Female A1221 (0.5) | Male A1221 (0.5) | 0.51 | 41.77 |
| Female A1221 (0.5) | Female A1221 (1.0) | 0.81 | 27.79 |
| Female A1221 (0.5) | Male A1221 (1.0) | 0.42 | 41.66 |
| Female A1221 (0.5) | Female Vehicle | 0.65 | 40.26 |
| Female A1221 (0.5) | Male Vehicle | 0.72 | 34.30 |
| Female A1221 (0.5) | Female EB | 0.26 | 46.96 |
| Female A1221 (0.5) | Male EB | 0.20 | 48.00 |
| Male A1221 (0.5) | Female A1221 (1.0) | 0.16 | 57.74 |
| Male A1221 (0.5) | Male A1221 (1.0) | 0.57 | 39.77 |
| Male A1221 (0.5) | Female Vehicle | 0.30 | 57.05 |
| Male A1221 (0.5) | Male Vehicle | 0.15 | 60.99 |
| Male A1221 (0.5) | Female EB | 0.08 | 68.77 |
| Male A1221 (0.5) | Male EB | 0.19 | 53.09 |
| Female A1221 (1.0) | Male A1221 (1.0) | 0.15 | 49.40 |
| Female A1221 (1.0) | Female Vehicle | 0.16 | 54.76 |
| Female A1221 (1.0) | Male Vehicle | 0.93 | 24.85 |
| Female A1221 (1.0) | Female EB | 0.75 | 28.77 |
| **Female A1221 (1.0)** | **Male EB** | **0.03** | **60.18** |
| Male A1221 (1.0) | Female Vehicle | 0.11 | 61.94 |
| Male A1221 (1.0) | Male Vehicle | 0.22 | 49.21 |
| Male A1221 (1.0) | Female EB | 0.07 | 57.44 |
| Male A1221 (1.0) | Male EB | 0.66 | 32.61 |
| Female Vehicle | Male Vehicle | 0.30 | 50.41 |
| **Female Vehicle** | **Female EB** | **0.04** | **69.55** |
| **Female Vehicle** | **Male EB** | **0.03** | **67.16** |
| Male Vehicle | Female EB | 0.88 | 28.77 |
| Male Vehicle | Male EB | 0.08 | 55.16 |
| **Female EB** | **Male EB** | **0.01** | **65.14** |

(Table 4.9 Continued on next page)

| B. Females Only | | | |
|---|---|---|---|
| Comparison | | Probability | Distance |
| A1221 (0.5) | A1221 (1.0) | 0.80 | 27.66 |
| A1221 (0.5) | Vehicle | 0.65 | 40.60 |
| A1221 (0.5) | EB | 0.32 | 46.35 |
| A1221 (1.0) | Vehicle | 0.12 | 56.94 |
| A1221 (1.0) | EB | 0.77 | 28.84 |
| **Vehicle** | **EB** | **0.04** | **70.32** |

| C. Males Only | | | |
|---|---|---|---|
| Comparison | | Probability | Distance |
| A1221 (0.5) | A1221 (1.0) | 0.59 | 39.91 |
| A1221 (0.5) | Vehicle | 0.20 | 60.40 |
| A1221 (0.5) | EB | 0.26 | 53.42 |
| A1221 (1.0) | Vehicle | 0.26 | 49.10 |
| A1221 (1.0) | EB | 0.68 | 31.99 |
| Vehicle | EB | 0.06 | 56.56 |

Table 4.9. Linear Discriminate Analysis

Systematic pairwise comparisons were made via linear discriminate analysis (LDA) of the entire dataset. Both probability and Mahalanobis distance values are shown for all animals (A), or for each sex separately (Females, B; Males, C). Instances of significant separation between compared behavioral datasets are shown in bold.

***Functional Landscape Analysis of Behaviors for Sex Differences***

Two variables, Time in Whole Arm and Number of Nose Touch Events were further analyzed by landscape analysis, chosen because the first represents an initial exploratory act in evaluating a conspecific (similar to appetitive behaviors in mating), and the second representing the most active engagement allowed animals (closest to the consummatory act). For Time in Whole Arm (Figure 4.4) there was a significant effect of sex for the EB group, with the female and male landscape profiles differing significantly ($p < 0.002$). The other 3 treatment groups did not differ by sex. For the Number of Nose Touch Events, similar analysis revealed that the A1221 (0.5) group was the only one with a significant sex difference ($p < 0.05$; Figure 4.5).

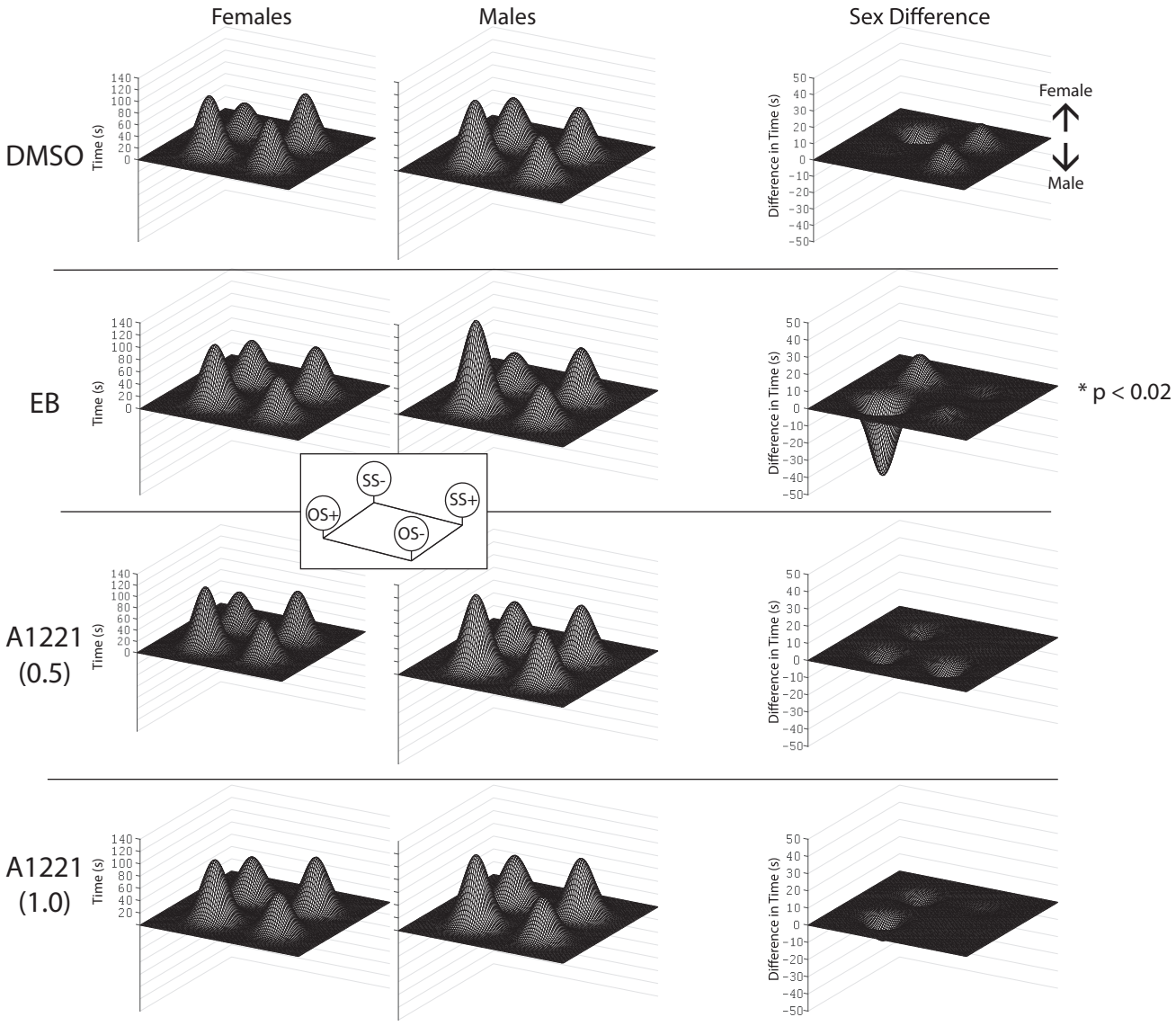



Figure 4.4 Time in Whole Arm

Time Spent in the Whole Arm, an appetitive behavior, is shown for females (left), males (middle), and the sex difference (right) as functional landscapes. For each sex, the height of each peak shows the absolute amount of time spent in the arm (seconds). For the sex difference, the y-axis shows the time differential, with an upward peak indicating F > M, and a downward valley indicating M > F. The only landscape profile that differed significantly between the sexes was the EB group. The positions of the four stimulus choices in each landscape are indicated by the inset.

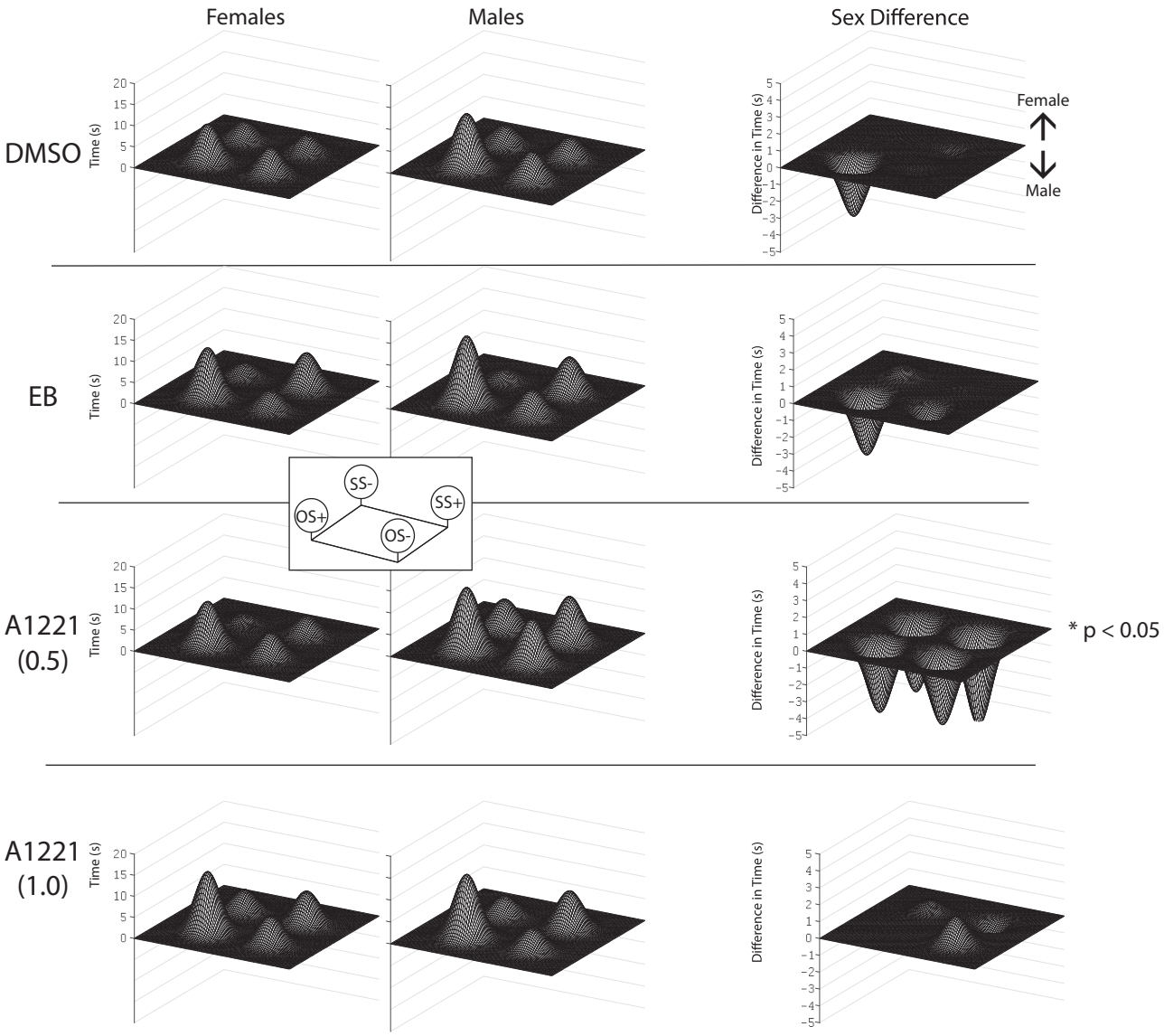



Figure 4.5. Number of Nose Touch Events

Number of Nose Touch Events (consummatory behavior) is shown for females (left), males (center), and the sex difference (right). Labels and analysis are the same as in Figure 4.4. A sex difference was found only for the A1221 (0.5) group.

## DISCUSSION

The concept that the EDCs are one of a number of potent environmental stressors has been gaining traction (Grandjean et al., 2015; Padmanabhan, Cardoso, & Puttabyatappa, 2016). The effects of these chemicals must be put in the broader context of the anthropogenic, natural, and social environments. Our focus on how prenatal PCB exposures change the trajectory of development adds to knowledge about a subset of these types of interactions, with an emphasis on evaluating outcomes in a more socially-relevant system. This study adds to the small but growing body of literature showing social behavior can be altered through developmental exposure to these PCBs (Jolous-Jamshidi, Cromwell, McFarland, & Meserve, 2010; Bell et al., 2015).

### Effects of Prenatal PCBs on Biological and Social Outcomes in the FourPlex

In nature, individuals differ by sex, reproductive status, age, and dominance hierarchy, among other traits. Rats are social animals living in communal settings with conspecifics of both sexes, a range of ages, health, and experiences. By expanding from the traditional two-choice to a four-choice test, we can systematically investigate the influence of more than one biologically-relevant factor on an experimental animal's social decision-making. In our apparatus, the experimental animal engages in motivated behavior, presumably driven by a desire to increase or decrease the distance between themselves and a stimulus animal. The choice to spend time near one stimulus is also inversely related to time away from other stimulus animals. Observing these behaviors within the context of endocrine disruption due to PCBs enables us to better understand

how low dose exposure to these compounds can lead to functional behavioral changes in adulthood.

In our study, there were no significant morphological differences observed throughout the development of these animals, consistent with prior work on similarly-treated animals (Gillette et al., 2017; Reilly et al., 2015). Thus, any alterations in behavior of these animals are likely not due to changes in the animal's health. In general, our results further show that irrespective of prenatal treatment, the experimental rats spent more time in parts of the apparatus in association with the OS+ stimulus animal. This result is consistent with the literature in two-choice models of a choice between same sex conspecifics of differing hormonal status: males prefer estrous (or estrogen-treated) females over non-estrous (or ovariectomized) females, and females prefer males with testosterone over castrated males (Xiao, Kondo, and Sakuma , 2004). Similarly, when presented with an opposite-sex binary choice, males prefer females, and females prefer males (Bakker, 2003; Carson, 2003; Henley, Nunez, & Clemens, 2011). Thus, the FourPlex is a sensitive tool for differentiating amongst multiple stimuli in a manner consistent with simpler systems.

Although the patterns of behavior in the FourPlex were largely preserved across prenatal treatment groups, there were several small but significant effects of prenatal treatment when considered in relationship to specific stimulus rats. These are best viewed in Table 9, which summarizes significant differences and/or LARGE effect sizes for treatment effects. In females, the only overall treatment effects were in the SS- arm (i.e. towards ovariectomized females): time spent in the remote part of the arm, and number of arm entries, was greater in EDC-exposed (especially A1221 (1.0)) than in vehicle females. We interpret this to mean that there is increased likelihood of the EDC females to affiliate with what is normally the least-salient stimulus. For males, the only significant

treatment effect was towards the OS- animal (ovariectomized female), limited to the number of stimulus explore events. In this case, A1221 (0.5) males had increased exploration compared to vehicle and EB rats. Although not significant, there was a trend ($p = 0.06$) for these animals to spend more time near the OS- rat's cage, and in the whole arm of that rat. It is noteworthy that the perception of and/or interaction with the ovariectomized female stimulus rat was the only one affected by treatment in both sexes. Finally, for experimental males in relationship to the OS+ rats (ovariectomized female plus estradiol) there were trends for total time, and time near the stimulus rat's cage, to be greater in EB than vehicle males. Thus, although wholesale behavioral changes were not caused by prenatal treatments, there are subtle shifts in interactions revealed in the FourPlex. While we do not know the mechanisms for these EDC effects, the finding that A1221 and EB have differential outcomes suggests that the former compound is acting by a non-estrogenic pathway. Furthermore, we point out that the prenatal EB treatment did not masculinize feminine behaviors, nor feminize masculine behavior. This is not unexpected given the low dose of EB and the short duration of treatment; in fact, our prior studies (Dickerson, Cunningham, & Gore, 2011; Steinberg, Walker, Juenger, Woller, & Gore, 2008; Walker, Goetz, & Gore, 2013; Gillette et al., 2017; Reilly et al., 2015) are consistent with the current finding of small EB effects on brain and behavior in this model.

**Prenatal EDCs Exacerbate Sex Differences in Behaviors**

The sexual dimorphism in behaviors in the FourPlex, or lack thereof, were influenced by prenatal EDC exposures (Table 9). When considering the OS+ rats – the most socially salient stimulus that was preferred by both sexes – prenatal EB treatment

introduced a novel sexual dimorphism, with males spending more time in the whole arm, time in the remote arm, and engaging in more stimulus explore and nose touch events. Sex differences and similarities in behaviors toward the SS- animal were the second-most commonly observed. For the number of stimulus explore and nose touch events, A1221 (0.5) treatment resulted in males having greater numbers of these events than females. In addition, numbers of stimulus explore events and arm entries toward the SS- rat was greater in EB females than males. The time spent in the remote portion of the SS- arm was only sexually dimorphic in the vehicle group. As for the SS+ group, a sex difference in time near the stimulus cage was found for vehicle and A1221 (1.0) rats (female > male) but not for the EB or A1221 (0.5) groups.

It is notable that most of these effects are in relationship to the OS+ and SS- groups, as these differ most in sociosexual valence. This underscores that the sensitivity of the FourPlex to discriminate the hierarchy of preference in rats is greatest for a hormonally-treated (or potentially gonadally intact) opposite sex rat, and lowest for a same-sex hormonally-deficient rat.

**FourPlex Results do not Mirror the Binary Choice Paradigm**

It is informative to consider the results for the vehicle group vis-à-vis validation of the FourPlex in control animals. For both sexes, the numbers of arm entries, time spent, and interactions with (especially nose touches) stimulus rats were highest toward the opposite-sex, hormone-treated rat. This result, while not surprising, shows that the layout of the apparatus is adequate for a rat to discriminate, and for an investigator to discern that discrimination in a 10-minute trial.

It is also informative to contrast the current results to those of our prior binary choice study, in which rats received identical treatments to those used here, but were tested differently in adulthood (Reilly et al., 2015). There, rats were given the opportunity to distinguish between two same-sex gonadectomized rats (no hormone), one familiar and one unfamiliar. Under those conditions, while all experimental rats showed the expected preference for a novel over a familiar rat, the differential was much greater in males than females, with the exception of the male A1221 (0.5) group. These animals were more similar in their behaviors to the females, showing a loss of the sexual dimorphism. By contrast, sex differences in the FourPlex arena were more likely to have an exaggerated dimorphism with treatment. Moreover, the magnitudes of changes in the FourPlex were, in general, smaller than in the two-choice test, suggesting a tempering of the outcomes in a more complex social setting.

**Ethological Implications**

It has been a long-lasting endeavor to balance ethological significance and experimental feasibility when studying behavior in laboratory animals. In practice, controlling the environment in dyadic choice models is favored over settings more representative of an animal's natural habitat due to the greater ease in simplifying behaviors in the former over the latter. However, advancements in technology have greatly aided efforts in creating environments capable of providing animals with more naturalistic setting. In particular, systems that allow for the automatic tracking of individual animals within a multi-animal, free-roaming environment have provided unique insights into the social hierarchy organization in a mouse model (So et al., 2015; Weissbrod et al., 2013). Automatic scoring of behaviors within such paradigms provide

means of discovering novel and objective metrics of social behavior. Hong et al. used a simultaneous video and depth camera setup in combination with computerized vision and supervised machine learning methods to gain unprecedented resolution (30 frames/second) of social interaction behavior between two strains of mice. This allowed the detection of extremely subtle differences in bout-length investigation previously undetectable through more standard methods (Hong et al., 2015).

It is notable that work in other fields have, on occasion, utilized 4+ choice paradigms. For example, a study on the effects of amphetamines on rat social behavior revealed that both treated and control rats would augment their aggregative tendency in proportion to the number of stimulus rats within the behavioral apparatus (Heimstra & McDonald, 1962). Studies on mate preference in rats have provided a female rat with four males and revealed differences in the dynamics of sexual selection when compared to binary choice models (Ferreira-Nuño et al., 2005). The field of cognitive neuroscience has many examples of learning-based tasks in radial arm mazes with multiple choices (Witty, Foster, Semple-Rowland, & Daniel, 2012), although in most cases the stimuli are objects or food rather than conspecifics. Nevertheless, the importance of mimicking the more realistic situation of multiple rather than binary possibilities underscores the potential application of the FourPlex to studies on endocrine disruption and other environmental perturbations.

# CHAPTER 5: THE EFFECTS OF GESTATIONAL EXPOSURE TO POLYCHLORINATED BIPHENYLS ON THE PRESENCE OF OXYTOCIN AND VASOPRESSIN IN THE PARAVENTRICULAR AND SUPRAOPTIC NUCLEI OF THE ADULT HYPOTHALAMUS


## ABSTRACT

Endocrine disrupting chemicals (EDCs) induce changes in hormone-sensitive tissues, causing responses that may lead to dysfunctional physiology. The effects of EDCs are especially influential during prenatal exposure, since the developing fetus is extremely sensitive to hormone-controlled tissue growth. There is a critical period of fetal development during which sex-steroids are particularly crucial in organizing and sustaining a properly developing nervous system in a sexually dimorphic manner. Furthermore, a resurgence of these sex-steroids during puberty has an activational effect on this neural circuitry, which accounts for sex differences in the neuronal control of reproductive physiology and behaviors in adulthood. Preliminary data have shown that polychlorinated biphenyl (PCB) exposure abolishes the preference toward a novel conspecific in a sexually dimorphic manner. The actions of the neuropeptides oxytocin (OT) and Vasopressin (AVP), critical to the manifestation of rodent social behavior, are subject to mediation and modulation via estrogenic processes. These experiments, which used the brains from behaviorally characterized animals, tested hypothesis that early-life exposure to PCBs may lead to changes to the oxytocin- or vasopressin-producing neurons in the hypothalamus. Looking at both female and male rats, no significant effects of treatment were found in the number or density of OT- or AVP-positive neurons. These results suggest that the PCB-mediated alterations in adult social behavior are not due to changes in the amount of oxytocin or vasopressin neurons in the paraventricular or

supraoptic nucleus. Further experimentation will be required to uncover the neurological bases for the observed changes.

## INTRODUCTION

Of the countless number of man-made chemicals that now exist in our environment, Endocrine disrupting chemicals (EDCs) pose a particularly persistent risk to those inhabiting the contaminated areas and beyond. The Endocrine Society's updated statement (Gore et al, 2015) defines EDCs as "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action", exposure to EDCs during critical periods of development have the potential to lead to lost-lasting effects. The developmental processes that occur during gestation, through birth, and into the early life are greatly influenced by both the presence or absence of endogenous sex steroids. Throughout all of life, but particularly during these sensitive periods, natural sex steroids organize and sustain a properly developing nervous system in a sexually dimorphic manner. Exposures to EDCs in early life can perturb these processes, changing how the brain develops, and causing functional behavioral changes later in adulthood.

The present study focuses on a ubiquitous EDC mixture of polychlorinated biphenyls (PCBs), which is a manmade compound that has contaminated the environment. Although the production was banned in the United States in 1979, this persistence of PCBs pose a continued risk. Experiments with mice and rats reveal that early exposure to these organochlorides interfere with brain mechanisms that modulate anxiety, reproductive, and social behaviors in adulthood. (Reilly et al. 2015, Bell et al. 2014, Gillette et al. 2017).

In mammals, social groups are characterized by high levels of complexity in the type/amount of social interactions amongst each other. In order for the manifestation of appropriate behavioral actions, the processing of social information needs to be precise for sociality to exist. This requires specific regulation of specific brain mechanisms associated with (1) recognition and (2) interpretation of various aspects of social information. Among the several neurobiological systems, neuroendocrine mechanisms appear to play a prominent role in social information processing. The brain distribution of oxytocin and vasopressin receptors and their genes have been linked to the presence or absence of monogamy and pair bonding in voles and peromyscus (Ross, HE, 2009). Oxytocin and vasopressin are, in turn, under the control of gonadal hormones. Sex steroid hormones mediate specific activation of different genes underlying a wide variety of social behaviors; even when these behaviors are ultimately regulated by different neurotransmitter systems in different brain regions (Choleris, 2008). One particular behavior essential for life in most social species is social recognition, which can be defined as the ability of an organism to distinguish between conspecifics such as a mate, an intruder, a subordinate or dominant member of the social hierarchy. Both oxytocin and vasopressin have been implicated in the processes governing social recognition (Lee HJ, 2008; Takayanagi Y, 2005; Engelmann M, 1994; Bielsky IF, 2005).

This manuscript aims to complement previously published findings that exposure to PCBs during gestation lead to sex- and dose-specific alterations of social behavior in adult rats (Reilly et al. 2016). Because the timing of gestational exposure in the present model coincides with the onset in the development of these two nonapeptides (Buijs, 1980), we hypothesized that the changes in behavior previously reported in these animals

may have been due to differences in the production of oxytocin and vasopressin at the main sites of synthesis, the PVN and SON (Figure 5.1). Presented here is an immunohistological investigation to identify if any changes in the synthesis of these nonapeptides may correlate with the changes we've seen in the behavior of these animals.

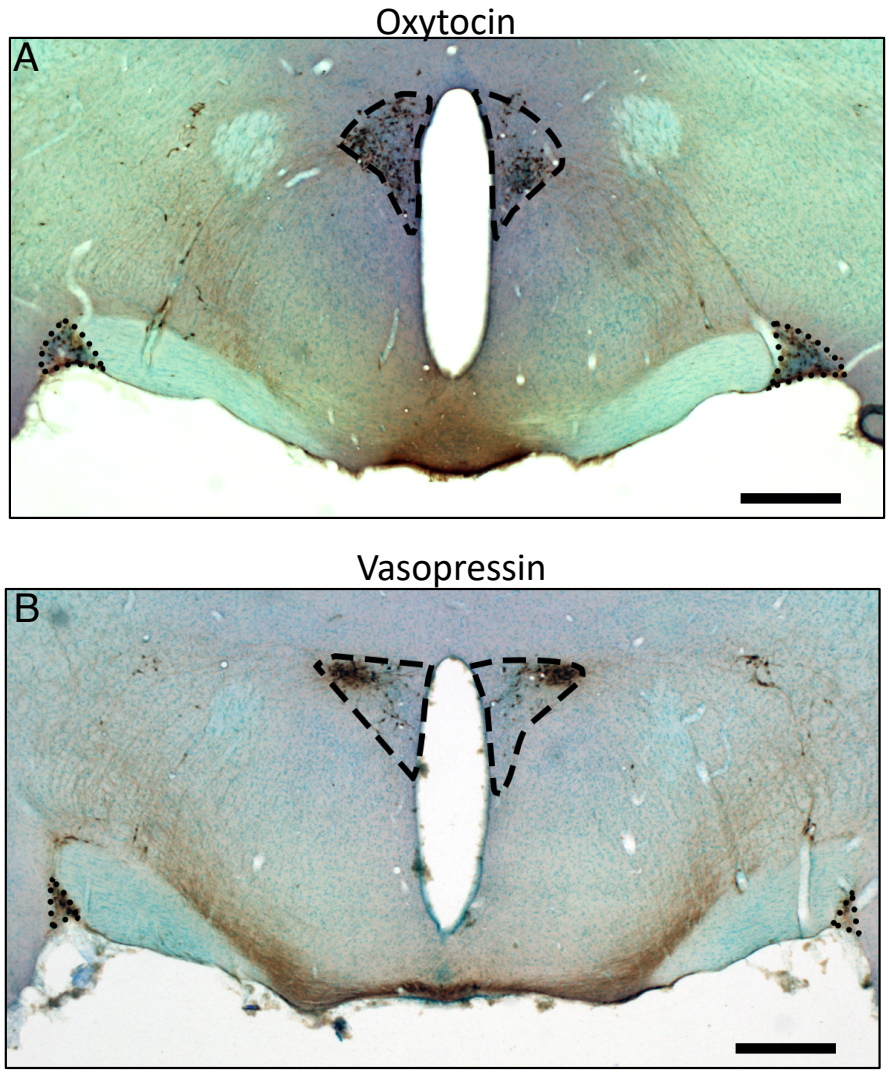



Figure 5.1. Oxytocin and Vasopressin Cell Bodies in The Paraventricular and Supraoptic Nuclei

Low-magnification coronal photomicrographs from a vehicle-treated male showing the two regions of interest: the paraventricular nucleus (dashed outline) and the supraoptic nucleus (dotted outline). This macro perspective shows the oxytocin(A)- and vasopressin(B)- positive cell bodies, detected by the DAB-peroxidase reaction. Tissues were counterstained with cresyl violet (nissl) staining. Scale bar = 500μm

## METHODS

### Animals and Husbandry

Young adult male and female Sprague-Dawley rats, aged approximately 3 months, were purchased from Harlan (Indianapolis, IN) for use in this study, which was conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Austin, following NIH guidelines. All animals were housed in same-sex groups of 2-3 inside of polycarbonate cages with ad-libitum access to water and a low-phytoestrogen chow (Harlan-Tekland Extruded 2019 Global Rodent diet). The colony was maintained at room temperature (21-22 C) on a partially reversed 12:12 light cycle (lights on at 12:00 am). Following a one-week acclimation period, females were smeared daily to determine estrous cyclicity. On days of proestrus, the virgin female was placed in a cage with a sexually experienced male to confirm receptivity. Then, the pair was left together overnight and a vaginal smear containing sperm served as the marker for embryonic day 0 (E0). Males were removed and the females were left singly housed for the duration of their pregnancy; nestlet bedding was provided several days prior to parturition on E21.

### Gestational Exposure to Endocrine Disrupting Chemicals

Pregnant dams were injected intraperitonally once on E16 and again with the same treatment on E18. The four experimental treatment groups were as follows: (a) vehicle (3% dimethylsulfoxide in sesame oil; negative control), (b) estradiol benzoate (EB 50 μg/kg; positive control for the estrogenic effects of PCBs), and the PCB mixture Aroclor 1221 at one of two concentrations: (c) (A1221, 0.5 mg/kg) or (d) (A1221, 1.0 mg/kg). For the duration of this manuscript, the four treatment groups will be referred to as vehicle, EB, A1221 (0.5), or A1221 (1.0), respectively. On the day of parturition (E21,

subsequently referred to as postnatal Day 0(P0)), pups were weighed and the anogenital distance (AGD) measured. To maintain an equal sex ratio across all groups, the litters were weaned to 4 males and 4 females. In addition to daily monitoring for signs of eye opening, weekly weights and AGD measurements occurred through weaning at P21. Initially, the animals were placed into same-sex groups, 4 animals per cage while the animals were monitored for pubertal development (vaginal opening in females, preputial separation in males). Postpubertal females were smeared daily and vaginal cytology was inspected under a microscope to determine and record estrous cyclicity for the duration of their lifetime. On P49, the same-sex groups were further divided into same-sex dyads maintained through the completion of the study. On P60, one animal from each of these cages was randomly selected for behavioral analyses, detailed in the following publications: Reilly et al 2015, Gillette et al, 2017. The total number of animals used to generate tissues was: Vehicle – 11 females, 10 males; EB – 9 females, 10 males; A1221 (0.5)- 9 females, 9 males; A1221 (1.0)- 8 females, 9 males.

**Euthanasia, Tissue Collection, and Processing.**

All animals were euthanized at ~P90; to control for cycle status, all females were euthanized on proestrus. Half of the animals were anesthetized with ketamine (AnimalHealth; 150 mg/kg) and xylazine (AnimalHealth; 30 mg/kg) and perfused with 4% paraformaldehyde (PFA) in PBS following lab protocols (Dickerson et al. 2011, Naugle et al. 2014, Kermath et al. 2014). The brains were postfixed overnight in 4% PFA at 4C and then stored in a sucrose cryoprotectant buffer at -20 for long-term storage. Tissues were blocked coronally and the region containing the hypothalamus was glued to a stage and sectioned on a Leica VT1000 vibrating microtome from Bregma -0.6 through Bregma 2.0 (Paxinos et al. 2007)

Two separate immunohistochemistry experiments were conducted utilized DAB immunostaining to quantify the number of oxytocin (OT) and vasopressin (AVP) neurons in a 1:5 tissue series, resulting in 5 sections for each animal.

***Immunohistochemistry***

Free floating sections were washed in PBS and subsequently quenched of any endogenous peroxidase in a 3:1 solution of methanol and 3% hydrogen peroxide in PBS for 20 minutes. Tissues were then incubated for 1 hour in a blocking solution containing 10% normal goal serum (NGS; Vector Laboratories), 0.5% Triton X (Vector Laboratories) in PBS. Incubation in primary antibody was done for oxytocin (Millipore MAB5296, mouse monoclonal at 1:20,000) or vasopressin (Immunostar 20069, rabbit polyclonal at 1:40,000) in 2% NGS at 4C for 48 hours. Tissues were washed in PBS and then incubated for 2 hours in a solution containing 2% NGS and secondary antibody (oxytocin: Vector Laboratories Biotinylated Goat Anti-Mouse IgG #BA-9200; vasopressin: Vector Laboratories Biotinylated Goat Anti-Rabbit IgG Cat#BA-1000) at 1:400 in PBS. Tissues were then placed in an avidin-biotin solution (ABC kit, Vector) for 1 hour, followed by 3,3′-Diaminobenzidine (DAB, Vector) reaction on ice for either 2 minutes (oxytocin) or 4 minutes (vasopressin). Between every step, tissues were washed with PBS; all reactions occurred at room temperature unless otherwise noted. The 5 section series per antibody per animal was mounted on a slide, counterstained with a cresyl violet (Nissl) solution, covered in DPX mountant (Sigma, Aldrich), and coverslipped.

***Cell counting.***

In order to assess the effects of treatment on the number of Oxytocin and Vasopressin neurons, all mounted sections were visualized on an Olympus BX61 microscope. Using Stereoinvestigator (MBF Bioscience, v10.0) the bilateral PVN and SON regional borders, as detected by the nissl stain, were outlined to calculate the area (μm); and all immunopositive neurons within the borders were counted. Analyses were done separately for the 5 sections collected from rostral to caudal (Representative series shown in Figure 5.2 and Figure 5.3 for oxytocin and vasopressin, respectively). For each section, based on the 40 μm tissue thickness, the regional density was calculated as:

$$Regional\ Density\left(\#\ immunopositive\frac{neurons}{\mu m^3}\right) = \frac{[Total\ \#\ of\ immunopositive\ neurons]}{[(\text{Total area}) * 40\ \mu\text{m}]}$$

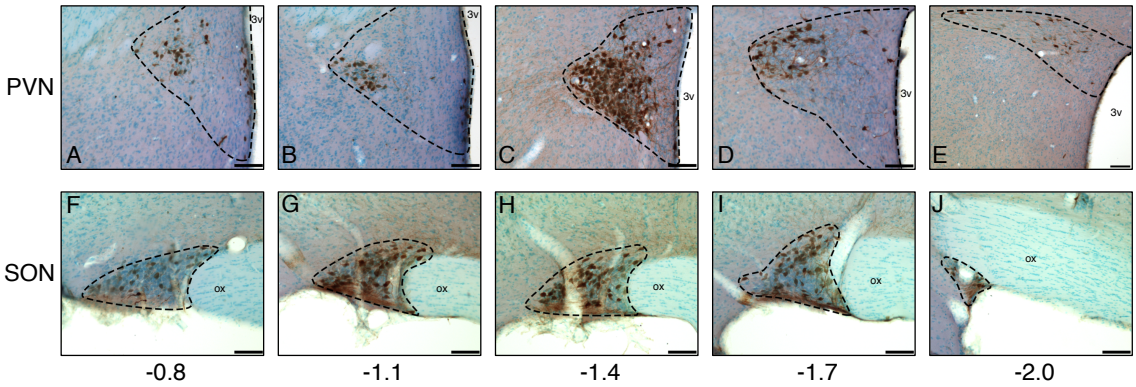



Figure 5.2. Oxytocin-positive Cell Bodies in the Paraventricular and Supraoptic Nuclei

Representative photomicrographs from a vehicle-treated female rat showing the rostral-to-caudal (left-to-right) distribution of oxytocin- positive cell bodies in the paraventricular nucleus (PVN; A-E) and supraoptic nucleus(SON; F-J), as detected by the DAB-peroxidase reaction. Both regions have been outlined. All tissues were counterstained with cresyl violet (nissl) staining. Abbreviations: 3v, third ventricle; ox, optic chiasm. Scale bar = 100μm

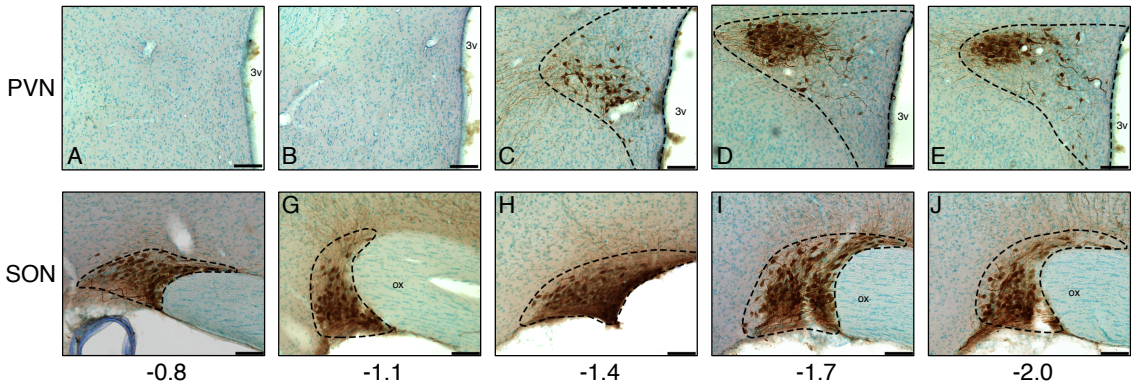



Figure 5.3. Vasopressin-positive Cell Bodies in the Paraventricular and Supraoptic nuclei

Representative photomicrographs from a vehicle-treated male rat showing the rostral-to-caudal (left-to-right) distribution of vasopressin-positive cell bodies in the paraventricular nucleus (PVN; A-E) and supraoptic nucleus(SON; F-J), as detected by the DAB-peroxidase reaction. Both regions have been outlined. No vasopressin staining was seen in the first two PVN sections of the experiment (shown in panels A and B). All tissues were counterstained with a cresyl violet (nissl) stain. Abbreviations: 3v, third ventricle; ox, optic chiasm. Scale bar = 100μm.

### *Statistical Analyses*

JMP Pro (v13.0) was used for statistical analyses. Datasets were examined for homogeneity of variance and normality, and ANOVA used when criteria were met. Because many data did not conform to the assumptions required for parametric analyses, the non-parametric Kruskal-Wallis test was performed to assess any treatment effects within the sexes, which were analyzed separately to evaluate any sex differences within each treatment via t-tests . The heterogeneity of the dataset limited the treatment comparisons to single rostral-caudal levels; Effect sizes were calculated for all comparisons made: partial-$\eta^2$ or $\varepsilon^2$, respectively, accompany the ANOVA/Kruskal-Wallis tests while Cohen's d effect sizes describe the sex differences within treatment. Interpretation of the effect size values for partial-$\eta^2$ and $\varepsilon^2$ were as follows: 0.01 = Small; 0.09 = Medium; 0.25 = Large. For Cohen's d: 0.2 = small; 0.5 = Medium; 0.8 = Large.

## RESULTS

### Total number of oxytocin- and vasopressin-immunoreactive cells for the entire series

### *Oxytocin*

In the PVN of females (Table 5.1A) and males (Table 5.1B), there were no significant effects of treatment on the total number of oxytocin-positive cell bodies counted in the experiment (Figure 5.4A). A trend for a sex difference in oxytocin neuron numbers in the vehicle groups (female > male) was found (Table 5.1C; $p = 0.06$); this had a Large effect size. For the SON, no significant effects of sex or treatment were observed (Figure 5.4B). A trend was found for a sex difference in the A1221(0.5) groups (male > female; $p = 0.06$), with a Large effect size.

***Vasopressin***

There were no significant effects of treatment in the total number of vasopressin-positive cell bodies within either the PVN or SON (Table 5.1; Figure 5.4C, 5.4D). One trend for a sex difference was found for vasopressin in PVN for the EB group (male > female; $p = 0.06$), with a Medium effect size.

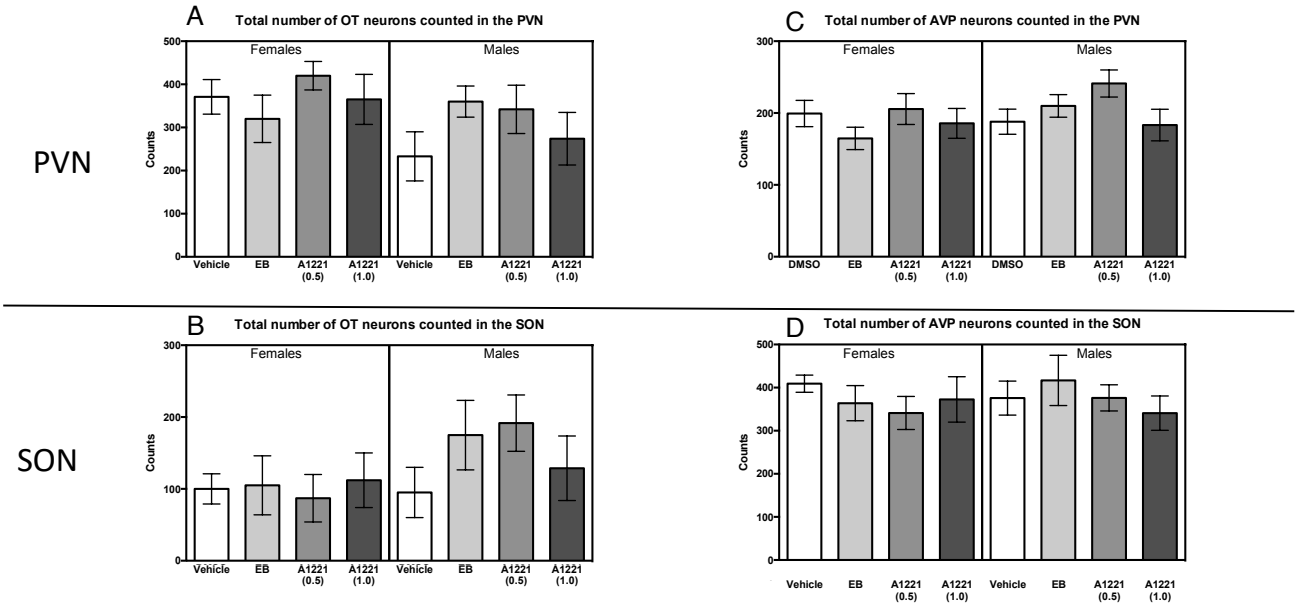



Figure 5.4. Total Number of Oxytocin- and Vasopressin-positive Cell Bodies in the Paraventricular and Supraoptic Nuclei

The total number of oxytocin- and vasopressin-positive cells counted in the paraventricular (PVN) and supraoptic nuclei (SON). There were no effects of treatment on the total number of cells detected in either region. The full statistical report can be found in Table 1. Means and SEM are shown.

A. Female Treatment Effects

| Target | Region | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|---|
| Oxytocin | PVN | Vehicle | 11 | 371 | ± | 40 | ANOVA | $F_{(3,34)} = 0.74$ | 0.54 | 0.06 |
| | | EB | 9 | 320 | ± | 55 | | | | |
| | | A1221 (0.5) | 9 | 420 | ± | 33 | | | | |
| | | A1221 (1.0) | 8 | 365 | ± | 58 | | | | |
| | SON | Vehicle | 11 | 100 | ± | 21 | KW | $X^2_{(3)} = 0.90$ | 0.83 | 0.03 |
| | | EB | 8 | 105 | ± | 41 | | | | |
| | | A1221 (0.5) | 9 | 87 | ± | 33 | | | | |
| | | A1221 (1.0) | 7 | 112 | ± | 38 | | | | |
| Vasopressin | PVN | Vehicle | 11 | 199 | ± | 18 | ANOVA | $F_{(3,33)} = 1.26$ | 0.45 | 0.08 |
| | | EB | 9 | 165 | ± | 15 | | | | |
| | | A1221 (0.5) | 8 | 206 | ± | 22 | | | | |
| | | A1221 (1.0) | 8 | 186 | ± | 21 | | | | |
| | SON | Vehicle | 11 | 409 | ± | 20 | ANOVA | $F_{(3,33)} = 0.62$ | 0.61 | 0.05 |
| | | EB | 9 | 364 | ± | 41 | | | | |
| | | A1221 (0.5) | 8 | 341 | ± | 38 | | | | |
| | | A1221 (1.0) | 8 | 373 | ± | 53 | | | | |

B. Male Treatment Effects

| Target | Region | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|---|
| Oxytocin | PVN | Vehicle | 10 | 233 | ± | 57 | ANOVA | $F_{(3,35)} = 1.30$ | 0.29 | 0.10 |
| | | EB | 10 | 360 | ± | 36 | | | | |
| | | A1221 (0.5) | 9 | 342 | ± | 56 | | | | |
| | | A1221 (1.0) | 9 | 274 | ± | 61 | | | | |
| | SON | Vehicle | 10 | 95 | ± | 35 | KW | $X^2_{(3)} = 3.62$ | 0.31 | 0.10 |
| | | EB | 10 | 175 | ± | 48 | | | | |
| | | A1221 (0.5) | 9 | 192 | ± | 39 | | | | |
| | | A1221 (1.0) | 9 | 129 | ± | 45 | | | | |
| Vasopressin | PVN | Vehicle | 9 | 188 | ± | 17 | ANOVA | $F_{(3,28)} = 2.04$ | 0.13 | 0.19 |
| | | EB | 8 | 210 | ± | 16 | | | | |
| | | A1221 (0.5) | 8 | 241 | ± | 19 | | | | |
| | | A1221 (1.0) | 6 | 183 | ± | 22 | | | | |
| | SON | Vehicle | 9 | 376 | ± | 39 | ANOVA | $F_{(3,29)} = 0.48$ | 0.70 | 0.05 |
| | | EB | 8 | 417 | ± | 58 | | | | |
| | | A1221 (0.5) | 8 | 376 | ± | 30 | | | | |
| | | A1221 (1.0) | 7 | 341 | ± | 40 | | | | |

(Table 5.1 continued on next page)

| C. Sex Effects within Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Target | Region | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| Oxytocin | PVN | Vehicle | 0.06 | 0.87 | Large | |
| | | EB | 0.55 | -0.28 | Small | |
| | | A1221 (0.5) | 0.26 | 0.56 | Medium | |
| | | A1221 (1.0) | 0.30 | 0.52 | Medium | |
| | SON | Vehicle | 0.91 | 0.05 | Small | |
| | | EB | 0.29 | -0.51 | Medium | |
| | | A1221 (0.5) | 0.06 | -0.97 | Large | |
| | | A1221 (1.0) | 0.78 | -0.14 | Small | |
| Vasopressin | PVN | Vehicle | 0.66 | 0.20 | Small | |
| | | EB | 0.06 | -0.62 | Medium | |
| | | A1221 (0.5) | 0.24 | -0.62 | Medium | |
| | | A1221 (1.0) | 0.94 | 0.04 | Small | |
| | SON | Vehicle | 0.46 | 0.35 | Small | |
| | | EB | 0.47 | -0.36 | Small | |
| | | A1221 (0.5) | 0.49 | -0.36 | Small | |
| | | A1221 (1.0) | 0.64 | 0.25 | Small | |

Table 5.1. Total Number of Cells Counted per Region

Total number of cumulative oxytocin- and vasopressin- positive cell bodies across the entire series is shown for Females (A) and Males (B). Data here, and in subsequent Tables (5.2-5.9), are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There were no differences due to treatment or sex.

**Rostral to caudal distribution of oxytocin- and vasopressin-immunoreactive cells**

***Oxytocin***

Because of the heterogeneity of cell number and density, the analysis was broken down from rostral to caudal in the PVN (Figure 5.5A). There were no effects of treatment in the females (Table 5.2A). Males were also not significantly affected (Table 5.2B), although a trend at was found ($p = 0.08$; medium effect size) at Bregma -0.8, presumably driven by the EB group. There were several significant sex differences (Table 5.2C) at specific parts of the series. At Bregma = -0.8, the A1221 (1.0) sexes differed significantly (female > male; $p = 0.02$; Large effect size). At both Bregma = -1.1 and -1.7, a sex difference (female > male; $p < 0.05$; Large effect sizes) was detected for the vehicle group. Finally, EB-treated animals at Bregma = -1.7 had a significant sex difference (female > male, $p = 0.03$; Large effect size). There were no significant effects of treatment in the density (Figure 5.6A) in the females (Table 5.3A) or males (Table 5.3B). Sex differences in the density of oxytocin-positive neurons within this region matched those for cell numbers, with the inclusion of an additional significant sex difference in the animals treated with A1221 (0.5) (Table 5.3C; $p = 0.03$; female > male; Large effect size).

A similar analysis was done for oxytocin in the SON, where no effects of treatment were observed in either sex (Females: Table 5.4A; Males: Table 5.4B) for total cell numbers, as well as when considered from rostral to caudal (Figure 5.5B). One significant sex difference (Table 5.4C) was observed at Bregma = -1.7 in the A1221 (0.5) group (male > female; $p = 0.02$; Large effect size). Several other instances of Large effect sizes between the sexes were observed but these not accompanied by statistical differences. When expressed as density (Figure 5.5B), there were no significant effects

of treatment in any of the females (Table 5.5A). In males (Table 5.5B), a significant effect of treatment was observed at Bregma = -2 ($p = 0.03$; Large effect size). Post hoc analysis indicated that this effect was driven by the difference between the A1221 (0.5) and A1221 (1.0) males (A1221(0.5) > A1221 (1.0); $p < 0.05$). The only sex difference (Table 5.5C) identified was also at Bregma -2.0 in the A1221 (0.5) group (male > female; $p = 0.01$; Large effect size). Any other instances of large effect sizes were not met with statistical significance.

***Vasopressin***

In the PVN was little-to-no immunolabeling in the rostral-most two sections of this series, therefore these data were not evaluated (Figure 5.5C). Analysis of the three remaining sections resulted in no significant differences due to treatment in females (Table 5.6A), although a trend ($p = 0.09$; large effect size) was found at Bregma = -2. For males (Table 6B), there were no treatment effects. There were also no significant sex differences in any of the treatment groups (Table 5.6C), despite several instances of an associated Large effect size. There were no significant effects of treatment on vasopressin density in females (Figure 5.6C; Table 75.A), and one trend for males at Bregma = -1.4; Large effect size; Table 5.7B). Analysis indicated a significant sex difference (Table 5.7C) in the A1221 (0.5) group (male > female; $p = 0.004$; Large effect size).

Unlike the PVN, vasopressin neurons in the SON were detectable across the entire rostral to caudal extent of the series (Figure 5.5D). In both female (Table 5.8A) and male (Table 5.8B) animals, there were no significant effects of treatment. Additionally, comparing the sexes within each treatment group yielded no significant sex differences in

any treatment groups across the series (Table 5.8C). Similar findings were made for vasopressin cell density in the SON (Table 5.9).

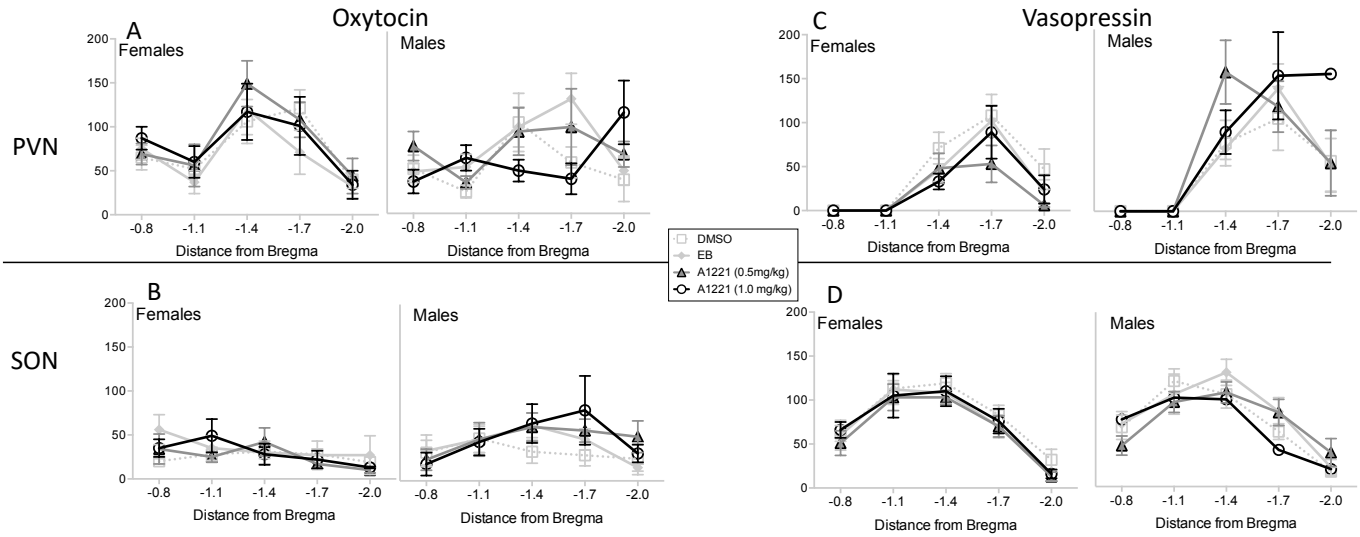



Figure 5.5. Rostral-to-caudal Distribution of Oxytocin and Vasopressin Immunopositive Neurons in the Paraventricular and Supraoptic Nuclei: Total Count

The total number of oxytocin- and vasopressin-positive cell bodies in the paraventricular (PVN) and supraoptic supraoptic nuclei (SON). Data are plotted rostral-to-caudal, respective to the 1:5 series, from Bregma -0.8 to -2.0. Full statistical report can be found in Tables 5.2, 5.4, 5.6, and 5.8. Means and SEM are shown.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 10 | 65 | ± | 14 | KW | $X^2_{(3)} = 1.26$ | 0.74 | 0.04 |
| | EB | 8 | 75 | ± | 12 | | | | |
| | A1221 (0.5) | 9 | 69 | ± | 12 | | | | |
| | A1221 (1.0) | 7 | 87 | ± | 13 | | | | |
| -1.1 | Vehicle | 10 | 52 | ± | 8 | KW | $X^2_{(3)} = 2.43$ | 0.49 | 0.07 |
| | EB | 9 | 37 | ± | 13 | | | | |
| | A1221 (0.5) | 8 | 56 | ± | 24 | | | | |
| | A1221 (1.0) | 7 | 60 | ± | 18 | | | | |
| -1.4 | Vehicle | 11 | 106 | ± | 25 | KW | $X^2_{(3)} = 1.56$ | 0.67 | 0.04 |
| | EB | 9 | 120 | ± | 29 | | | | |
| | A1221 (0.5) | 9 | 149 | ± | 26 | | | | |
| | A1221 (1.0) | 8 | 117 | ± | 32 | | | | |
| -1.7 | Vehicle | 11 | 121 | ± | 21 | KW | $X^2_{(3)} = 3.06$ | 0.31 | 0.10 |
| | EB | 9 | 71 | ± | 25 | | | | |
| | A1221 (0.5) | 9 | 108 | ± | 20 | | | | |
| | A1221 (1.0) | 7 | 101 | ± | 33 | | | | |
| -2 | Vehicle | 11 | 38 | ± | 8 | KW | $X^2_{(3)} = 1.14$ | 0.77 | 0.03 |
| | EB | 7 | 32 | ± | 13 | | | | |
| | A1221 (0.5) | 9 | 44 | ± | 20 | | | | |
| | A1221 (1.0) | 7 | 34 | ± | 16 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 10 | 49 | ± | 15 | KW | $X^2_{(3)} = 2.86$ | 0.41 | 0.08 |
| | EB | 10 | 46 | ± | 9 | | | | |
| | A1221 (0.5) | 8 | 74 | ± | 16 | | | | |
| | A1221 (1.0) | 7 | 35 | ± | 13 | | | | |
| -1.1 | Vehicle | 9 | 24 | ± | 7 | KW | $X^2_{(3)} = 4.209$ | 0.24 | 0.12 |
| | EB | 10 | 51 | ± | 14 | | | | |
| | A1221 (0.5) | 9 | 34 | ± | 7 | | | | |
| | A1221 (1.0) | 9 | 61 | ± | 14 | | | | |
| -1.4 | Vehicle | 8 | 100 | ± | 32 | KW | $X^2_{(3)} = 0.99$ | 0.80 | 0.03 |
| | EB | 10 | 95 | ± | 22 | | | | |
| | A1221 (0.5) | 9 | 90 | ± | 26 | | | | |
| | A1221 (1.0) | 7 | 47 | ± | 12 | | | | |
| -1.7 | Vehicle | 10 | 56 | ± | 17 | KW | $X^2_{(3)} = 6.78$ | 0.08 | 0.18 |
| | EB | 10 | 126 | ± | 28 | | | | |
| | A1221 (0.5) | 9 | 95 | ± | 42 | | | | |
| | A1221 (1.0) | 9 | 38 | ± | 17 | | | | |
| -2 | Vehicle | 7 | 37 | ± | 24 | KW | $X^2_{(3)} = 3.94$ | 0.27 | 0.12 |
| | EB | 9 | 47 | ± | 16 | | | | |
| | A1221 (0.5) | 8 | 65 | ± | 14 | | | | |
| | A1221 (1.0) | 9 | 111 | ± | 35 | | | | |

(Table 5.2 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | 0.43 | 0.36 | Small | |
| | EB | 0.08 | 0.90 | Large | |
| | A1221 (0.5) | 0.81 | -0.12 | Medium | |
| | A1221 (1.0) | **0.02** | **1.52** | **Large** | **F>M** |
| -1.1 | Vehicle | **0.02** | **1.20** | **Large** | **F>M** |
| | EB | 0.48 | -0.33 | Small | |
| | A1221 (0.5) | 0.39 | 0.45 | Small | |
| | A1221 (1.0) | 0.98 | -0.01 | Small | |
| -1.4 | Vehicle | 0.88 | 0.07 | Small | |
| | EB | 0.50 | 0.32 | Small | |
| | A1221 (0.5) | 0.13 | 0.75 | Medium | |
| | A1221 (1.0) | 0.07 | 1.03 | Large | |
| -1.7 | Vehicle | **0.03** | **1.04** | **Large** | **F>M** |
| | EB | 0.17 | -0.66 | Medium | |
| | A1221 (0.5) | 0.78 | 0.13 | Small | |
| | A1221 (1.0) | 0.12 | 0.88 | Large | |
| -2 | Vehicle | 0.97 | 0.02 | Small | |
| | EB | 0.48 | -0.36 | Small | |
| | A1221 (0.5) | 0.40 | -0.42 | Small | |
| | A1221 (1.0) | 0.07 | -0.95 | Large | |

Table 5.2. Number of Oxytocin-positive Cell Bodies in the Paraventricular Nucleus Across the Series

Total number of oxytocin- positive cell bodies counted in the paraventricular nucleus with respect to each section in the series is shown for Females (A) and Males (B). Data here, and in subsequent Tables 5.3-5.9, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with

directionality of the effect. There were no differences due to treatment. Three significant sex differences were found and are indicated. M=male, F=female.

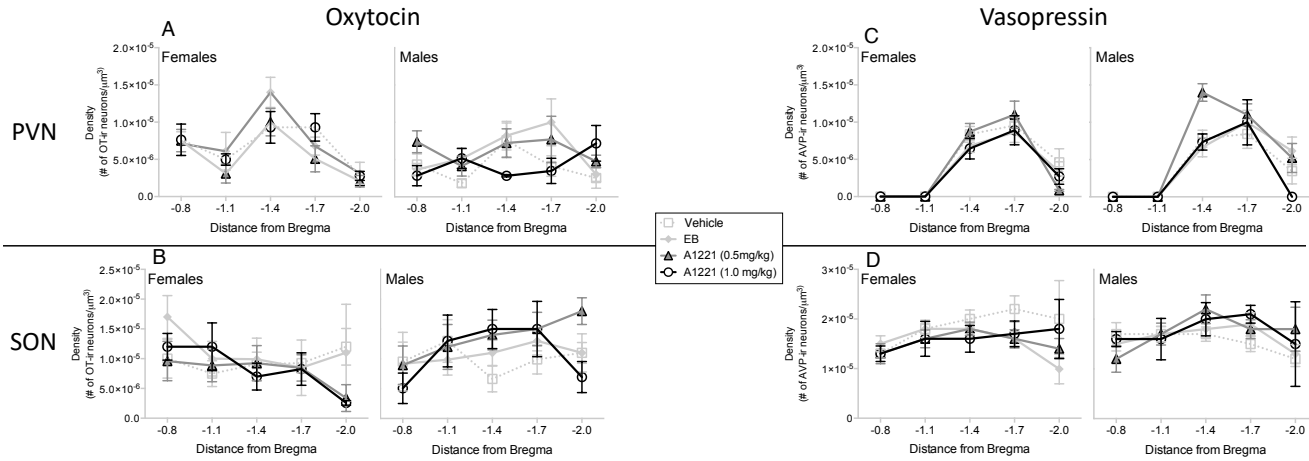



Figure 5.6. Rostral-to-caudal Distribution of Oxytocin and Vasopressin Immunopositive Neurons in the Paraventricular and Supraoptic Nuclei: Density

The density of oxytocin- and vasopressin-positive cell bodies per regional volume (per $\mu^3$) in the paraventricular (PVN) and supraoptic supraoptic nuclei (SON). Data are plotted rostral-to-caudal, respective to the 1:5 series, from Bregma -0.8 to -2.0. Full statistical report can be found in Tables 5.3, 5.5, 5.7, and 5.9. Means and SEM are shown.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 10 | 7.6E-06 | ± | 2.1E-06 | KW | $X^2_{(3)} = 0.18$ | 0.98 | 0.01 |
| | EB | 8 | 7.5E-06 | ± | 1.5E-06 | | | | |
| | A1221 (0.5) | 8 | 7.1E-06 | ± | 1.6E-06 | | | | |
| | A1221 (1.0) | 6 | 7.5E-06 | ± | 1.3E-06 | | | | |
| -1.1 | Vehicle | 10 | 5.0E-06 | ± | 0.8E-06 | KW | $X^2_{(3)} = 4.30$ | 0.23 | 0.13 |
| | EB | 9 | 3.1E-06 | ± | 1.3E-06 | | | | |
| | A1221 (0.5) | 7 | 6.1E-06 | ± | 2.5E-06 | | | | |
| | A1221 (1.0) | 7 | 6.1E-06 | ± | 2.1E-06 | | | | |
| -1.4 | Vehicle | 11 | 9.3E-06 | ± | 2.1E-06 | KW | $X^2_{(3)} = 3.20$ | 0.36 | 0.09 |
| | EB | 9 | 1.0E-05 | ± | 1.8E-06 | | | | |
| | A1221 (0.5) | 9 | 1.4E-05 | ± | 2.0E-06 | | | | |
| | A1221 (1.0) | 8 | 1.1E-05 | ± | 3.6E-06 | | | | |
| -1.7 | Vehicle | 11 | 9.3E-06 | ± | 1.8E-06 | KW | $X^2_{(3)} = 2.89$ | 0.41 | 0.08 |
| | EB | 9 | 5.1E-06 | ± | 1.7E-06 | | | | |
| | A1221 (0.5) | 9 | 6.8E-06 | ± | 1.2E-06 | | | | |
| | A1221 (1.0) | 7 | 7.3E-06 | ± | 2.2E-06 | | | | |
| -2 | Vehicle | 10 | 2.8E-06 | ± | 0.6E-06 | KW | $X^2_{(3)} = 1.35$ | 0.72 | 0.04 |
| | EB | 7 | 2.1E-06 | ± | 0.8E-06 | | | | |
| | A1221 (0.5) | 9 | 3.1E-06 | ± | 1.5E-06 | | | | |
| | A1221 (1.0) | 7 | 2.7E-06 | ± | 1.0E-06 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 10 | 4.3E-06 | ± | 1.4E-06 | KW | $X^2_{(3)} = 3.94$ | 0.27 | 0.12 |
| | EB | 9 | 3.6E-06 | ± | 0.8E-06 | | | | |
| | A1221 (0.5) | 8 | 7.4E-06 | ± | 1.5E-06 | | | | |
| | A1221 (1.0) | 7 | 2.8E-06 | ± | 1.3E-06 | | | | |
| -1.1 | Vehicle | 8 | 1.8E-06 | ± | 0.5E-06 | KW | $X^2_{(3)} = 4.11$ | 0.25 | 0.12 |
| | EB | 9 | 5.1E-06 | ± | 1.3E-06 | | | | |
| | A1221 (0.5) | 8 | 4.1E-06 | ± | 1.3E-06 | | | | |
| | A1221 (1.0) | 9 | 5.2E-06 | ± | 1.3E-06 | | | | |
| -1.4 | Vehicle | 8 | 7.57E-06 | ± | 2.3E-06 | KW | $X^2_{(3)} = 1.72$ | 0.63 | 0.05 |
| | EB | 10 | 8.21E-06 | ± | 1.9E-06 | | | | |
| | A1221 (0.5) | 9 | 7.23E-06 | ± | 1.9E-06 | | | | |
| | A1221 (1.0) | 7 | 2.83E-06 | ± | 0.2E-06 | | | | |
| -1.7 | Vehicle | 10 | 4.1E-06 | ± | 1.4E-06 | KW | $X^2_{(3)} = 4.72$ | 0.19 | 0.13 |
| | EB | 10 | 1.0E-05 | ± | 3.1E-06 | | | | |
| | A1221 (0.5) | 9 | 7.7E-06 | ± | 3.1E-06 | | | | |
| | A1221 (1.0) | 9 | 3.5E-06 | ± | 1.7E-06 | | | | |
| -2 | Vehicle | 7 | 2.5E-06 | ± | 1.4E-06 | KW | $X^2_{(3)} = 3.99$ | 0.26 | 0.12 |
| | EB | 9 | 3.0E-06 | ± | 0.9E-06 | | | | |
| | A1221 (0.5) | 8 | 4.8E-06 | ± | 0.8E-06 | | | | |
| | A1221 (1.0) | 9 | 7.2E-06 | ± | 2.4E-06 | | | | |

(Table 5.3 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | 0.21 | 0.58 | Medium | |
| | EB | 0.04 | 1.12 | Large | |
| | A1221 (0.5) | 0.90 | -0.06 | Small | |
| | A1221 (1.0) | **0.03** | **1.36** | **Large** | **F>M** |
| -1.1 | Vehicle | **0.004** | **1.57** | **Large** | **F>M** |
| | EB | 0.28 | -0.53 | Medium | |
| | A1221 (0.5) | 0.51 | 0.36 | Small | |
| | A1221 (1.0) | 0.73 | 0.71 | Medium | |
| -1.4 | Vehicle | 0.59 | 0.18 | Small | |
| | EB | 0.51 | 0.31 | Small | |
| | A1221 (0.5) | **0.03** | **1.15** | **Large** | **F>M** |
| | A1221 (1.0) | 0.07 | 1.15 | Large | |
| -1.7 | Vehicle | **0.04** | **0.98** | **Large** | **F>M** |
| | EB | 0.16 | -0.62 | Medium | |
| | A1221 (0.5) | 0.79 | -0.13 | Small | |
| | A1221 (1.0) | 0.19 | 0.71 | Medium | |
| -2 | Vehicle | 0.87 | 0.08 | Small | |
| | EB | 0.45 | -0.38 | Small | |
| | A1221 (0.5) | 0.35 | -0.46 | Small | |
| | A1221 (1.0) | 0.11 | -0.84 | Large | |

Table 5.3. Oxytocin Staining Density in the Paraventricular Nucleus Across the Series

The density of ocytocin-positive cell bodies by regional volume (per $\mu 3$) in the paraventricular nucleus with respect to each section in the series is shown here. Data here, and in subsequent Tables 5.4-5.9, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There were no differences due to treatment. The four significant sex differences found are indicated . M=male, F=female.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 6 | 20 | ± | 3 | KW | $X^2_{(3)} = 1.26$ | 0.42 | 0.13 |
| | EB | 5 | 56 | ± | 17 | | | | |
| | A1221 (0.5) | 6 | 34 | ± | 17 | | | | |
| | A1221 (1.0) | 6 | 35 | ± | 10 | | | | |
| -1.1 | Vehicle | 10 | 28 | ± | 8 | KW | $X^2_{(3)} = 1.13$ | 0.77 | 0.04 |
| | EB | 7 | 35 | ± | 14 | | | | |
| | A1221 (0.5) | 6 | 25 | ± | 6 | | | | |
| | A1221 (1.0) | 5 | 49 | ± | 19 | | | | |
| -1.4 | Vehicle | 11 | 31 | ± | 8 | KW | $X^2_{(3)} = 0.61$ | 0.89 | 0.02 |
| | EB | 7 | 30 | ± | 13 | | | | |
| | A1221 (0.5) | 7 | 42 | ± | 16 | | | | |
| | A1221 (1.0) | 7 | 28 | ± | 12 | | | | |
| -1.7 | Vehicle | 10 | 28 | ± | 6 | KW | $X^2_{(3)} = 1.40$ | 0.71 | 0.06 |
| | EB | 2 | 27 | ± | 16 | | | | |
| | A1221 (0.5) | 6 | 17 | ± | 5 | | | | |
| | A1221 (1.0) | 5 | 22 | ± | 10 | | | | |
| -2 | Vehicle | 4 | 19 | ± | 6 | KW | $X^2_{(3)} = 0.94$ | 0.81 | 0.09 |
| | EB | 2 | 27 | ± | 22 | | | | |
| | A1221 (0.5) | 3 | 10 | ± | 3 | | | | |
| | A1221 (1.0) | 2 | 13 | ± | 1 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 6 | 32 | ± | 14 | KW | $X^2_{(3)} = 1.19$ | 0.76 | 0.05 |
| | EB | 8 | 34 | ± | 18 | | | | |
| | A1221 (0.5) | 4 | 24 | ± | 12 | | | | |
| | A1221 (1.0) | 5 | 19 | ± | 13 | | | | |
| -1.1 | Vehicle | 5 | 48 | ± | 16 | KW | $X^2_{(3)} = 0.42$ | 0.94 | 0.02 |
| | EB | 9 | 47 | ± | 15 | | | | |
| | A1221 (0.5) | 7 | 47 | ± | 19 | | | | |
| | A1221 (1.0) | 7 | 44 | ± | 15 | | | | |
| -1.4 | Vehicle | 8 | 33 | ± | 13 | KW | $X^2_{(3)} = 3.06$ | 0.38 | 0.10 |
| | EB | 10 | 63 | ± | 14 | | | | |
| | A1221 (0.5) | 9 | 61 | ± | 16 | | | | |
| | A1221 (1.0) | 5 | 65 | ± | 22 | | | | |
| -1.7 | Vehicle | 7 | 29 | ± | 12 | KW | $X^2_{(3)} = 3.78$ | 0.29 | 0.16 |
| | EB | 7 | 47 | ± | 12 | | | | |
| | A1221 (0.5) | 7 | 57 | ± | 13 | | | | |
| | A1221 (1.0) | 3 | 80 | ± | 39 | | | | |
| -2 | Vehicle | 2 | 25 | ± | 18 | KW | $X^2_{(3)} = 3.26$ | 0.35 | 0.16 |
| | EB | 7 | 15 | ± | 4 | | | | |
| | A1221 (0.5) | 7 | 50 | ± | 18 | | | | |
| | A1221 (1.0) | 6 | 31 | ± | 10 | | | | |

(Table 5.4 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | 0.41 | -0.51 | Medium | |
| | EB | 0.40 | 0.49 | Small | |
| | A1221 (0.5) | 0.62 | 0.31 | Small | |
| | A1221 (1.0) | 0.39 | 0.56 | Medium | |
| -1.1 | Vehicle | 0.29 | -0.67 | Medium | |
| | EB | 0.57 | -0.28 | Small | |
| | A1221 (0.5) | 0.30 | -0.59 | Medium | |
| | A1221 (1.0) | 0.84 | 0.12 | Small | |
| -1.4 | Vehicle | 0.90 | -0.06 | Small | |
| | EB | 0.10 | -0.84 | Large | |
| | A1221 (0.5) | 0.42 | -0.41 | Small | |
| | A1221 (1.0) | 0.19 | -0.90 | Large | |
| -1.7 | Vehicle | 0.96 | -0.03 | Small | |
| | EB | 0.42 | -0.72 | Medium | |
| | A1221 (0.5) | **0.02** | **-1.56** | **Large** | **M>F** |
| | A1221 (1.0) | 0.27 | -1.16 | Large | |
| -2 | Vehicle | 0.80 | -0.31 | Small | |
| | EB | 0.68 | 0.52 | Medium | |
| | A1221 (0.5) | 0.07 | -1.18 | Large | |
| | A1221 (1.0) | 0.12 | -1.09 | Large | |

Table 5.4. Number of Oxytocin-positive Cell Bodies in the Supraoptic Nucleus Across the Series

Table 4. Total number of oxytocin- positive cell bodies counted in the supraoptic nucleus with respect to each section in the series is shown for Females (A) and Males (B). Data here, and in subsequent Tables 5.5-5.9, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There were no differences due to treatment. The one significant sex difference that was found is indicated. M=male, F=female.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 6 | 1.0E-05 | ± | 3.3E-06 | KW | $X^2_{(3)} = 2.63$ | 0.45 | 0.12 |
| | EB | 5 | 1.7E-05 | ± | 3.6E-06 | | | | |
| | A1221 (0.5) | 6 | 9.6E-06 | ± | 3.3E-06 | | | | |
| | A1221 (1.0) | 6 | 1.2E-05 | ± | 2.2E-06 | | | | |
| -1.1 | Vehicle | 10 | 7.5E-06 | ± | 2.2E-06 | KW | $X^2_{(3)} = 1.38$ | 0.71 | 0.05 |
| | EB | 7 | 1.0E-05 | ± | 2.9E-06 | | | | |
| | A1221 (0.5) | 6 | 8.9E-06 | ± | 2.7E-06 | | | | |
| | A1221 (1.0) | 5 | 1.2E-05 | ± | 4.0E-06 | | | | |
| -1.4 | Vehicle | 11 | 9.1E-06 | ± | 2.0E-06 | KW | X2(3) = 0.61 | 0.89 | 0.02 |
| | EB | 7 | 9.9E-06 | ± | 3.6E-06 | | | | |
| | A1221 (0.5) | 7 | 9.2E-06 | ± | 3.0E-06 | | | | |
| | A1221 (1.0) | 7 | 7.0E-06 | ± | 2.2E-06 | | | | |
| -1.7 | Vehicle | 10 | 9.3E-06 | ± | 1.6E-06 | KW | X2(3) = 0.29 | 0.96 | 0.01 |
| | EB | 2 | 8.5E-06 | ± | 4.7E-06 | | | | |
| | A1221 (0.5) | 6 | 8.4E-06 | ± | 2.2E-06 | | | | |
| | A1221 (1.0) | 5 | 8.3E-06 | ± | 2.7E-06 | | | | |
| -2 | Vehicle | 4 | 1.2E-05 | ± | 3.1E-06 | KW | X2(3) = 3.79 | 0.28 | 0.04 |
| | EB | 2 | 1.1E-05 | ± | 8.1E-06 | | | | |
| | A1221 (0.5) | 2 | 3.4E-06 | ± | 2.2E-06 | | | | |
| | A1221 (1.0) | 2 | 2.6E-06 | ± | 0.4E-06 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 6 | 9.5E-06 | ± | 4.9E-06 | KW | $X^2_{(3)}$ =0.82 | 0.85 | 0.04 |
| | EB | 8 | 9.2E-06 | ± | 3.6E-06 | | | | |
| | A1221 (0.5) | 4 | 8.9E-06 | ± | 3.2E-06 | | | | |
| | A1221 (1.0) | 5 | 5.1E-06 | ± | 2.5E-06 | | | | |
| -1.1 | Vehicle | 5 | 1.3E-05 | ± | 3.6E-06 | KW | $X^2_{(3)}$ =0.89 | 0.83 | 0.03 |
| | EB | 9 | 9.9E-06 | ± | 2.6E-06 | | | | |
| | A1221 (0.5) | 7 | 1.2E-05 | ± | 3.8E-06 | | | | |
| | A1221 (1.0) | 7 | 1.3E-05 | ± | 4.3E-06 | | | | |
| -1.4 | Vehicle | 8 | 6.63E-06 | ± | 2.2E-06 | KW | $X^2_{(3)}$ =5.69 | 0.13 | 0.18 |
| | EB | 10 | 11.0E-06 | ± | 2.1E-06 | | | | |
| | A1221 (0.5) | 9 | 14.0E-06 | ± | 2.5E-06 | | | | |
| | A1221 (1.0) | 5 | 15.0E-06 | ± | 3.3E-06 | | | | |
| -1.7 | Vehicle | 7 | 9.9E-06 | ± | 2.4E-06 | KW | $X^2_{(3)}$ =2.07 | 0.56 | 0.09 |
| | EB | 7 | 13.0E-06 | ± | 1.7E-06 | | | | |
| | A1221 (0.5) | 7 | 15.0E-06 | ± | 2.8E-06 | | | | |
| | A1221 (1.0) | 3 | 15.0E-06 | ± | 4.6E-06 | | | | |
| -2 | Vehicle | 2 | 11.0E-06 | ± | 3.2E-06 | KW | $X^2_{(3)}$ =8.93 | **0.03** | 0.43 |
| | EB | 7 | 11.0E-06 | ± | 1.7E-06 | | | | |
| | A1221 (0.5) | 7 | 18.0E-06 | ± | 2.2E-06 | | | | |
| | A1221 (1.0) | 6 | 6.9E-06 | ± | 2.6E-06 | | | | |

(Table 5.5 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | 0.92 | 0.05 | Small | |
| | EB | 0.16 | 0.86 | Large | |
| | A1221 (0.5) | 0.89 | 0.09 | Small | |
| | A1221 (1.0) | 0.07 | 1.24 | Large | |
| -1.1 | Vehicle | 0.23 | -0.74 | Medium | |
| | EB | 0.88 | 0.02 | Small | |
| | A1221 (0.5) | 0.57 | -0.37 | Small | |
| | A1221 (1.0) | 0.89 | -0.10 | Small | |
| -1.4 | Vehicle | 0.42 | 0.39 | Small | |
| | EB | 0.71 | -0.14 | Small | |
| | A1221 (0.5) | 0.28 | -0.62 | Medium | |
| | A1221 (1.0) | 0.10 | -1.20 | Large | |
| -1.7 | Vehicle | 0.83 | -0.11 | Small | |
| | EB | 0.50 | -0.81 | Large | |
| | A1221 (0.5) | 0.09 | -1.01 | Large | |
| | A1221 (1.0) | 0.26 | -0.94 | Large | |
| -2 | Vehicle | 0.76 | 0.19 | Small | |
| | EB | 0.99 | 0.00 | Small | |
| | A1221 (0.5) | **0.01** | **-3.08** | **Large** | M > F |
| | A1221 (1.0) | 0.16 | -0.97 | Large | |

Table 5.5. Oxytocin Staining Density in the Supraoptic Nucleus Across the Series

Table 5. The density of oxytocin-positive cell bodies by regional volume (per $\mu 3$) in the supraoptic nucleus with respect to each section in the series is shown here. Data here, and in subsequent Tables 5.6-5.9, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. One significant effect of treatment was found in males in the last section (Bregma -2.0). The one significant sex difference found is indicated. M=male, F=female.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 0 | N/A | ± | N/A | N/A | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.1 | Vehicle | 0 | N/A | ± | N/A | N/A | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.4 | Vehicle | 7 | 71 | ± | 18 | KW | $X^2_{(3)} = 2.31$ | 0.51 | 0.08 |
| | EB | 8 | 46 | ± | 13 | | | | |
| | A1221 (0.5) | 7 | 48 | ± | 17 | | | | |
| | A1221 (1.0) | 7 | 33 | ± | 9 | | | | |
| -1.7 | Vehicle | 11 | 108 | ± | 24 | KW | $X^2_{(3)} = 4.24$ | 0.24 | 0.12 |
| | EB | 9 | 102 | ± | 18 | | | | |
| | A1221 (0.5) | 8 | 53 | ± | 21 | | | | |
| | A1221 (1.0) | 8 | 89 | ± | 30 | | | | |
| -2 | Vehicle | 6 | 47 | ± | 23 | KW | $X^2_{(3)} = 6.60$ | 0.09 | 0.37 |
| | EB | 5 | 21 | ± | 14 | | | | |
| | A1221 (0.5) | 4 | 6 | ± | 2 | | | | |
| | A1221 (1.0) | 4 | 24 | ± | 16 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 0 | N/A | ± | N/A | N/A | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.1 | Vehicle | 0 | N/A | ± | N/A | N/A | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.4 | Vehicle | 7 | 75 | ± | 25 | KW | $X^2_{(3)} = 4.17$ | 0.24 | 0.22 |
| | EB | 6 | 69 | ± | 12 | | | | |
| | A1221 (0.5) | 3 | 153 | ± | 35 | | | | |
| | A1221 (1.0) | 4 | 87 | ± | 24 | | | | |
| -1.7 | Vehicle | 7 | 102 | ± | 35 | KW | $X^2_{(3)} = 1.35$ | 0.72 | 0.05 |
| | EB | 7 | 135 | ± | 27 | | | | |
| | A1221 (0.5) | 8 | 115 | ± | 28 | | | | |
| | A1221 (1.0) | 4 | 149 | ± | 48 | | | | |
| -2 | Vehicle | 3 | 55 | ± | 34 | KW | $X^2_{(3)} = 2.46$ | 0.48 | 0.22 |
| | EB | 5 | 51 | ± | 29 | | | | |
| | A1221 (0.5) | 4 | 53 | ± | 36 | | | | |
| | A1221 (1.0) | 1 | 151 | ± | N/A | | | | |

(Table 5.6 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | N/A | N/A | N/A | N/A |
| | EB | N/A | N/A | N/A | N/A |
| | A1221 (0.5) | N/A | N/A | N/A | N/A |
| | A1221 (1.0) | N/A | N/A | N/A | N/A |
| -1.1 | Vehicle | N/A | N/A | N/A | N/A |
| | EB | N/A | N/A | N/A | N/A |
| | A1221 (0.5) | N/A | N/A | N/A | N/A |
| | A1221 (1.0) | N/A | N/A | N/A | N/A |
| -1.4 | Vehicle | 0.90 | -0.07 | Small | |
| | EB | 0.23 | -0.67 | Medium | |
| | A1221 (0.5) | 0.07 | -1.99 | Large | |
| | A1221 (1.0) | 0.11 | -1.43 | Large | |
| -1.7 | Vehicle | 0.89 | 0.07 | Small | |
| | EB | 0.32 | -0.53 | Medium | |
| | A1221 (0.5) | 0.10 | -0.89 | Medium | |
| | A1221 (1.0) | 0.33 | -0.67 | Medium | |
| -2 | Vehicle | 0.86 | -0.14 | Small | |
| | EB | 0.38 | -0.60 | Medium | |
| | A1221 (0.5) | 0.28 | -0.93 | Large | |
| | A1221 (1.0) | N/A | N/A | N/A | N/A |

Table 5.6. Number of Vasopressin-positive Cell Bodies in the Paraventricular Nucleus Across the Series

Total number of vasopressin- positive cell bodies counted in the paraventricular nucleus with respect to each section in the series is shown for Females (A) and Males (B). Data here, and in subsequent Tables 5.7-5.9, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There were no differences due to treatment or sex. M=male, F=female.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 0 | N/A | ± | N/A | KW | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.1 | Vehicle | 0 | N/A | ± | N/A | KW | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.4 | Vehicle | 7 | 7.7E-06 | ± | 1.5E-06 | KW | $X^2_{(3)} = 1.34$ | 0.72 | 0.05 |
| | EB | 8 | 6.0E-06 | ± | 1.2E-06 | | | | |
| | A1221 (0.5) | 7 | 5.8E-06 | ± | 1.4E-06 | | | | |
| | A1221 (1.0) | 6 | 4.6E-06 | ± | 1.4E-06 | | | | |
| -1.7 | Vehicle | 11 | 8.1E-06 | ± | 1.5E-06 | KW | $X^2_{(3)} = 4.01$ | 0.26 | 0.11 |
| | EB | 9 | 8.6E-06 | ± | 1.4E-06 | | | | |
| | A1221 (0.5) | 8 | 4.7E-06 | ± | 1.7E-06 | | | | |
| | A1221 (1.0) | 8 | 6.7E-06 | ± | 2.2E-06 | | | | |
| -2 | Vehicle | 6 | 4.6E-06 | ± | 1.8E-06 | KW | $X^2_{(3)} = 4.01$ | 0.16 | 0.29 |
| | EB | 4 | 3.3E-06 | ± | 1.3E-06 | | | | |
| | A1221 (0.5) | 3 | 0.8E-06 | ± | 0.1E-06 | | | | |
| | A1221 (1.0) | 4 | 2.7E-06 | ± | 1.0E-06 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 0 | N/A | ± | N/A | N/A | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.1 | Vehicle | 0 | N/A | ± | N/A | N/A | N/A | N/A | N/A |
| | EB | 0 | N/A | ± | N/A | | | | |
| | A1221 (0.5) | 0 | N/A | ± | N/A | | | | |
| | A1221 (1.0) | 0 | N/A | ± | N/A | | | | |
| -1.4 | Vehicle | 7 | 6.97E-06 | ± | 1.5E-06 | KW | $X^2_{(3)} = 7.30$ | 0.06 | **0.38** |
| | EB | 6 | 7.76E-06 | ± | 0.7E-06 | | | | |
| | A1221 (0.5) | 3 | 14.0eE06 | ± | 1.2E-06 | | | | |
| | A1221 (1.0) | 4 | 7.96E-06 | ± | 1.2E-06 | | | | |
| -1.7 | Vehicle | 6 | 8.5E-06 | ± | 2.3E-06 | KW | $X^2_{(3)} = 0.41$ | 0.94 | 0.02 |
| | EB | 7 | 9.8E-06 | ± | 1.8E-06 | | | | |
| | A1221 (0.5) | 8 | 9.8E-06 | ± | 1.9E-06 | | | | |
| | A1221 (1.0) | 4 | 10.0E-06 | ± | 3.0E-06 | | | | |
| -2 | Vehicle | 3 | 4.6E-06 | ± | 1.8E-06 | KW | $X^2_{(3)} = 2.72$ | 0.44 | 0.25 |
| | EB | 4 | 5.3E-06 | ± | 2.3E-06 | | | | |
| | A1221 (0.5) | 4 | 5.2E-06 | ± | 1.9E-06 | | | | |
| | A1221 (1.0) | 1 | 12.0E-06 | ± | N/A | | | | |

(Table 5.7 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | N/A | N/A | N/A | |
| | EB | N/A | N/A | N/A | |
| | A1221 (0.5) | N/A | N/A | N/A | |
| | A1221 (1.0) | N/A | N/A | N/A | |
| -1.1 | Vehicle | N/A | N/A | N/A | |
| | EB | N/A | N/A | N/A | |
| | A1221 (0.5) | N/A | N/A | N/A | |
| | A1221 (1.0) | N/A | N/A | N/A | |
| -1.4 | Vehicle | 0.73 | 0.19 | Small | |
| | EB | 0.23 | -0.66 | Medium | |
| | A1221 (0.5) | **0.004** | **-2.69** | **Large** | **M>F** |
| | A1221 (1.0) | 0.11 | -1.13 | Large | |
| -1.7 | Vehicle | 0.89 | -0.07 | Small | |
| | EB | 0.60 | -0.27 | Small | |
| | A1221 (0.5) | 0.06 | -1.01 | Large | |
| | A1221 (1.0) | 0.38 | -0.54 | Medium | |
| -2 | Vehicle | 0.99 | 0.00 | Small | |
| | EB | 0.50 | -0.52 | Medium | |
| | A1221 (0.5) | 0.11 | -1.59 | Large | |
| | A1221 (1.0) | N/A | N/A | N/A | |

Table 5.7. Vasopressin Staining Density in the Paraventricular Nucleus Across the Series

The density of vasopressin-positive cell bodies by regional volume (per $\mu 3$) in the paraventricular nucleus with respect to each section in the series is shown here. Data here, and in subsequent Tables 5.8 and 5.9, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. No significant effects of treatment were found. The one significant sex difference is indicated. M=male, F=female.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 11 | 62 | ± | 9 | KW | $X^2_{(3)} = 2.31$ | 0.86 | 0.02 |
| | EB | 9 | 60 | ± | 17 | | | | |
| | A1221 (0.5) | 8 | 51 | ± | 14 | | | | |
| | A1221 (1.0) | 8 | 66 | ± | 9 | | | | |
| -1.1 | Vehicle | 11 | 113 | ± | 9 | KW | $X^2_{(3)} = 0.45$ | 0.93 | 0.01 |
| | EB | 9 | 112 | ± | 17 | | | | |
| | A1221 (0.5) | 8 | 103 | ± | 15 | | | | |
| | A1221 (1.0) | 8 | 105 | ± | 25 | | | | |
| -1.4 | Vehicle | 11 | 119 | ± | 11 | KW | $X^2_{(3)} = 2.11$ | 0.55 | 0.06 |
| | EB | 9 | 108 | ± | 12 | | | | |
| | A1221 (0.5) | 8 | 103 | ± | 8 | | | | |
| | A1221 (1.0) | 8 | 110 | ± | 17 | | | | |
| -1.7 | Vehicle | 11 | 83 | ± | 11 | KW | $X^2_{(3)} = 1.01$ | 0.80 | 0.03 |
| | EB | 9 | 70 | ± | 13 | | | | |
| | A1221 (0.5) | 8 | 70 | ± | 12 | | | | |
| | A1221 (1.0) | 8 | 76 | ± | 14 | | | | |
| -2 | Vehicle | 11 | 32 | ± | 12 | KW | $X^2_{(3)} = 0.74$ | 0.86 | 0.02 |
| | EB | 9 | 14 | ± | 6 | | | | |
| | A1221 (0.5) | 8 | 13 | ± | 5 | | | | |
| | A1221 (1.0) | 8 | 16 | ± | 5 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 9 | 68 | ± | 14 | KW | $X^2_{(3)} = 2.21$ | 0.53 | 0.07 |
| | EB | 8 | 72 | ± | 14 | | | | |
| | A1221 (0.5) | 8 | 47 | ± | 11 | | | | |
| | A1221 (1.0) | 7 | 77 | ± | 22 | | | | |
| -1.1 | Vehicle | 9 | 121 | ± | 14 | KW | $X^2_{(3)} = 1.14$ | 0.77 | 0.04 |
| | EB | 8 | 106 | ± | 23 | | | | |
| | A1221 (0.5) | 8 | 97 | ± | 12 | | | | |
| | A1221 (1.0) | 7 | 102 | ± | 26 | | | | |
| -1.4 | Vehicle | 9 | 106 | ± | 16 | KW | $X^2_{(3)} = 1.51$ | 0.68 | 0.05 |
| | EB | 8 | 131 | ± | 15 | | | | |
| | A1221 (0.5) | 8 | 108 | ± | 12 | | | | |
| | A1221 (1.0) | 7 | 100 | ± | 20 | | | | |
| -1.7 | Vehicle | 9 | 63 | ± | 9 | KW | $X^2_{(3)} = 4.88$ | 0.18 | 0.16 |
| | EB | 8 | 86 | ± | 16 | | | | |
| | A1221 (0.5) | 8 | 85 | ± | 15 | | | | |
| | A1221 (1.0) | 7 | 42 | ± | 16 | | | | |
| -2 | Vehicle | 9 | 17 | ± | 5 | KW | $X^2_{(3)} = 4.88$ | 0.40 | 0.09 |
| | EB | 8 | 22 | ± | 9 | | | | |
| | A1221 (0.5) | 8 | 39 | ± | 16 | | | | |
| | A1221 (1.0) | 7 | 20 | ± | 15 | | | | |

(Table 5.8 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | 0.72 | -0.17 | Small | |
| | EB | 0.56 | -0.29 | Small | |
| | A1221 (0.5) | 0.83 | 0.11 | Small | |
| | A1221 (1.0) | 0.65 | -0.25 | Small | |
| -1.1 | Vehicle | 0.63 | -0.22 | Small | |
| | EB | 0.83 | 0.11 | Small | |
| | A1221 (0.5) | 0.76 | 0.16 | Small | |
| | A1221 (1.0) | 0.93 | 0.05 | Small | |
| -1.4 | Vehicle | 0.50 | 0.31 | Small | |
| | EB | 0.25 | -0.58 | Medium | |
| | A1221 (0.5) | 0.77 | -0.15 | Small | |
| | A1221 (1.0) | 0.71 | 0.20 | Small | |
| -1.7 | Vehicle | 0.19 | 0.61 | Medium | |
| | EB | 0.46 | -0.37 | Small | |
| | A1221 (0.5) | 0.46 | -0.38 | Small | |
| | A1221 (1.0) | 0.14 | 0.82 | Large | |
| -2 | Vehicle | 0.28 | 0.48 | Small | |
| | EB | 0.48 | -0.36 | Small | |
| | A1221 (0.5) | 0.15 | -0.79 | Medium | |
| | A1221 (1.0) | 0.83 | -0.12 | Small | |

Table 5.8. Number of Vasopressin-positive Cell Bodies in the Supraoptic Nucleus Across the Series

Total number of vasopressin- positive cell bodies counted in the supraoptic nucleus with respect to each section in the series is shown for Females (A) and Males (B). Data here, and in subsequent Table 5.9, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. There were no differences due to treatment or sex. M=male, F=female.

A. Female Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 11 | 1.3E-05 | ± | 0.9E-06 | KW | $X^2_{(3)} = 1.14$ | 0.77 | 0.04 |
| | EB | 7 | 1.5E-05 | ± | 1.6E-06 | | | | |
| | A1221 (0.5) | 7 | 1.3E-05 | ± | 2.0E-06 | | | | |
| | A1221 (1.0) | 8 | 1.3E-05 | ± | 1.6E-06 | | | | |
| -1.1 | Vehicle | 11 | 1.8E-05 | ± | 1.1E-06 | KW | $X^2_{(3)} = 1.14$ | 0.67 | 0.04 |
| | EB | 9 | 1.8E-05 | ± | 1.8E-06 | | | | |
| | A1221 (0.5) | 8 | 1.6E-05 | ± | 1.8E-06 | | | | |
| | A1221 (1.0) | 8 | 1.6E-05 | ± | 3.5E-06 | | | | |
| -1.4 | Vehicle | 11 | 2.0E-05 | ± | 1.8E-06 | KW | $X^2_{(3)} = 2.28$ | 0.51 | 0.07 |
| | EB | 9 | 1.8E-05 | ± | 1.0E-06 | | | | |
| | A1221 (0.5) | 8 | 1.8E-05 | ± | 1.4E-06 | | | | |
| | A1221 (1.0) | 7 | 1.6E-05 | ± | 2.7E-06 | | | | |
| -1.7 | Vehicle | 11 | 2.2E-05 | ± | 2.6E-06 | KW | $X^2_{(3)} = 3.11$ | 0.37 | 0.09 |
| | EB | 8 | 1.6E-05 | ± | 1.5E-06 | | | | |
| | A1221 (0.5) | 8 | 1.6E-05 | ± | 1.8E-06 | | | | |
| | A1221 (1.0) | 8 | 1.7E-05 | ± | 2.6E-06 | | | | |
| -2 | Vehicle | 9 | 2.0E-05 | ± | 7.7E-06 | KW | $X^2_{(3)} = 1.38$ | 0.71 | 0.05 |
| | EB | 7 | 1.0E-05 | ± | 3.0E-06 | | | | |
| | A1221 (0.5) | 6 | 1.4E-05 | ± | 2.1E-06 | | | | |
| | A1221 (1.0) | 6 | 1.8E-05 | ± | 5.9E-06 | | | | |

B. Male Treatment Effects

| Distance from Bregma | Treatment | N | Mean (#/$\mu^3$) | | SEM | Test | F (df) for ANOVA; $X^2$ (df) for KW | P-value | $\eta_p^2$ for ANOVA; $\varepsilon^2$ for KW |
|---|---|---|---|---|---|---|---|---|---|
| -0.8 | Vehicle | 8 | 1.7E-05 | ± | 2.3E-06 | KW | $X^2_{(3)} = 3.82$ | 0.28 | 0.13 |
| | EB | 8 | 1.5E-05 | ± | 1.4E-06 | | | | |
| | A1221 (0.5) | 8 | 1.2E-05 | ± | 2.7E-06 | | | | |
| | A1221 (1.0) | 7 | 1.6E-05 | ± | 1.5E-06 | | | | |
| -1.1 | Vehicle | 9 | 1.7E-05 | ± | 1.6E-06 | KW | $X^2_{(3)} = 0.33$ | 0.95 | 0.01 |
| | EB | 8 | 1.7E-05 | ± | 1.5E-06 | | | | |
| | A1221 (0.5) | 8 | 1.7E-05 | ± | 2.2E-06 | | | | |
| | A1221 (1.0) | 7 | 1.6E-05 | ± | 4.2E-06 | | | | |
| -1.4 | Vehicle | 9 | 1.70E-05 | ± | 1.4E-06 | KW | $X^2_{(3)} = 1.19$ | 0.76 | 0.04 |
| | EB | 8 | 1.80E-05 | ± | 1.6E-06 | | | | |
| | A1221 (0.5) | 8 | 2.20E-05 | ± | 2.9E-06 | | | | |
| | A1221 (1.0) | 6 | 2.00E-05 | ± | 3.3E-06 | | | | |
| -1.7 | Vehicle | 9 | 1.5E-05 | ± | 1.5E-06 | KW | $X^2_{(3)} = 4.86$ | 0.18 | 0.17 |
| | EB | 8 | 1.9E-05 | ± | 2.3E-06 | | | | |
| | A1221 (0.5) | 8 | 1.8E-05 | ± | 1.9E-06 | | | | |
| | A1221 (1.0) | 5 | 2.1E-05 | ± | 1.8E-06 | | | | |
| -2 | Vehicle | 7 | 1.2E-05 | ± | 1.5E-06 | KW | $X^2_{(3)} = 1.42$ | 0.70 | 0.06 |
| | EB | 7 | 1.3E-05 | ± | 1.9E-06 | | | | |
| | A1221 (0.5) | 6 | 1.8E-05 | ± | 4.4E-06 | | | | |
| | A1221 (1.0) | 3 | 1.5E-05 | ± | 8.5E-06 | | | | |

(Table 5.9 continued on next page)

| C. Sex Effects within Treatment | | | | | |
|---|---|---|---|---|---|
| Distance from Bregma | Treatment | P-Value | Cohen's d | Effect Size | Directionality |
| -0.8 | Vehicle | 0.14 | -0.79 | Medium | |
| | EB | 0.93 | 0.00 | Small | |
| | A1221 (0.5) | 0.73 | 0.15 | Small | |
| | A1221 (1.0) | 0.18 | -0.71 | Medium | |
| -1.1 | Vehicle | 0.675 | 0.24 | Small | |
| | EB | 0.63 | 0.21 | Small | |
| | A1221 (0.5) | 0.66 | -0.18 | Small | |
| | A1221 (1.0) | 0.88 | 0.00 | Small | |
| -1.4 | Vehicle | 0.33 | 0.57 | Medium | |
| | EB | 0.95 | 0.00 | Small | |
| | A1221 (0.5) | 0.25 | -0.63 | Medium | |
| | A1221 (1.0) | 0.30 | -0.52 | Medium | |
| -1.7 | Vehicle | 0.06 | 1.00 | Large | |
| | EB | 0.37 | -0.55 | Medium | |
| | A1221 (0.5) | 0.37 | -0.38 | Small | |
| | A1221 (1.0) | 0.23 | -0.68 | Medium | |
| -2 | Vehicle | 0.34 | 0.48 | Small | |
| | EB | 0.46 | -0.47 | Small | |
| | A1221 (0.5) | 0.42 | -0.47 | Small | |
| | A1221 (1.0) | 0.23 | 0.20 | Small | |

Table 5.9. Vasopressin Staining Density in the Supraoptic Nucleus Across the Series

The density of vasopressin-positive cell bodies by regional volume (per $\mu 3$) in the supraoptic nucleus with respect to each section in the series is shown here. Data here, and in subsequent Tables x-x, are shown as mean + SEM. Statistical tests used, values and degrees of freedom, and effect sizes are indicated (SMALL = 0.01; MEDIUM = 0.09; LARGE = 0.25). (C) The statistics for within-treatment sex differences and Cohen's d effect sizes are shown (SMALL = 0.2; MEDIUM = 0.5; LARGE = 0.8) with directionality of the effect. No significant effects of treatment or sex were found. M=male, F=female.

## DISCUSSION

The purpose of the present study was to evaluate the effects of gestational exposure to PCBs on the oxytocin- and -vasopressin producing neurons within two hypothalamic brain regions, the paraventricular and supraoptic nuclei. Previous work in the lab identified changes in the social and anxiety-related behavior of these same animals (Reilly, 2015; Gillette 2017). In tests of sociality, the binary choice model identified that females in the EB and A1221 groups did not display the expected preference for the novel stimulus animal; this was also seen in the male A1221 (0.5 mg/kg) group. Although it is not possible to attribute this outcome to any one specific mechanism, there exists precedent in the literature to suggest that an animal's ability to recognize a conspecific is impaired by deficiencies in the oxytocin and vasopressin signaling system.

In this report, we present evidence of no significant differences in the expression of oxytocin- or vasopressin-positive cell bodies within the paraventricular or supraoptic nucleus in animals exposed to low dosages of the PCB mixture A1221. Although the timing of exposure coincided with the ontogeny of these two peptides, the exogenous effects during gestation did not drive any changes to the production of either peptide in the adult brain. Thus, the sex- and dose-specific changes observed in the social behavior of these animals (Reilly, 2016) may exist downstream of the peptidergic activity of these two regions.

Many experiments identifying the role of oxytocin (OT) and vasopressin (AVP) in social recognition have done so through the manipulation of the respective receptors

away from the sites of production. For example, the actions of OT on it's receptor (OTR) in the medial amygdala (MeA) has been determined to play a critical role in an animal's ability to display behavior consistent with social recognition (Ferguson, 2000). The MeA is the site of convergence of socially-relevant olfactory systems. Infusion of OT into the MeA of OT-knockout mice restored social recognition (Beauchamp, 2003). Studies in AVP-deficient Battleboro rats have shown changes in social recognition as well (Feifel, 2009). In contrast to the actions of OT on this behavior, knockout studies in mice have determined that the actions of AVP on social recognition lie at the level of the lateral septum (LS), where vasopressin receptor subtype 1a (AVP1aR)-KO mice fail to display behavior suggesting familiarity to a stimulus animal (Bielsky 2004). Re-expression of the gene encoding for AVP1aR in the LS was sufficient to reinstate social recognition in the knockout mice (Bielsky, 2005). Moreover, sex steroid hormones modulate the activity of the peptides at these regions and, in the case of OT's action at the MeA, serve as a requirement for normal function. Although both estrogen receptor alpha (ERα) and beta (ERβ) have been implicated in social behavior (Clipperton, 2006), ERα is required for the induction of the OTR at the MeA (Cushing, 2008; Spiteri, 2011).

Without evidence of changes in the synthesis of oxytocin or vasopressin in the PVN or SON, it's possible that the alterations in behavior observed in our animals were mediated by changes to the target regions of the peptides, such as the LS or MeA. The degree of estrogenic modulation in these regions presents a sensitivity to endocrine disruption by PCBs; particularly if exposure occurs during development of these regions during gestation. Having no reliable antibody against the specific OT or AVP receptors, such a theory is not feasibly addressed through immunohistochemical methods. Rather, future work will investigate the MeA for changes in oxytocin, vasopressin, and the

receptors to see if changes to the genes that encode for these proteins is altered by gestational exposure to PCBs, presumably via estrogenic mechanisms.

# CHAPTER 6: SUMMARY, IMPLICATIONS, AND FUTURE DIRECTIONS

The purpose of this dissertation was to develop an in-depth behavioral analysis of animals exposed to EDCs during gestation. By observing both sexes in an assortment of paradigms that model social behavior, I was able to determine how the sexes differed in their behavior and whether these differences presented differential susceptibilities to endocrine disruption. In general, each chapter had at least one experimental factor that was shared with another chapter. This internal consistency presented the opportunity to compare and contrast the results, aiding in the interpretation of the outcomes.

## SUMMARY OF FINDINGS

### Gestational EDCs do Not Alter the Social Preference

The results of chapters 2 and 3 give me confidence in determining that gestational exposure to PCBs or VIN do not alter the general affiliative behaviors of the animals. Regardless of sex or treatment, all animals displayed a clear-cut preference to spent time investigating and interacting with the stimulus cage containing a stimulus animal compared to the empty stimulus cage, as is consistent with the literature in rats. Interestingly, sex differences that were present in Chapter 2 were either absent or reversed in Chapter 3. Specifically, Chapter 2 determined that, although there were no alterations of the Social Preference due to treatment, there was a main effect of sex in the (1) latency to explore the stimulus animal (M>F) and (2) the total amount of time spent nose touching (M>F). In chapter 3, we did not see a significant effect of sex in the total time spent nose touching. Comparing the outcomes of the two chapters, I noticed that this was driven by the females of Chapter 3 engaging in more nose touching behavior with the stimuli used. In contrast to the same-sex gonadectomized stimulus animals used in

Chapter 2, Chapter 3 used same-sex intact stimulus animals for the binary choice test. This suggests that the intact stimulus elicited more nose touching from the females in all treatment groups within this social setting. An alternate, though not definitively mutually exclusive, explanation between the discordance in this sex difference may also lie in the difference in exposure durations. While Chapter 2 animals were subject to the effects of EDC during the time when the hypothalamus was going through sexual differentiation (E16 and E18), the animals in Chapter 3 received treatment much earlier (beginning on E08) and for a longer duration (daily until E18). This paradigm also encompasses the development of the fetal gonads, which play a crucial role in the subsequent sexual differentiation of the brain. It is tempting to suggest that exposure to PCBs at an a point prior to the sexual differentiation of the brain altered the developmental trajectory such that the processes mediating the sex differences were altered.

**Gestational EDCs alter the Novelty Preference in a Sex- Dose- and Duration-Specific Manner**

In contrast to the unaffected Social Preference as determined by the Sociability test, Chapters 2 and 3 both saw sex- and dose-specific alterations to Novelty Preference. In Chapter 2, A1221 disrupted the novelty preference in females at the 1.0 mg/kg dose and males at the 0.5 mg/kg dose; this phenotype was observed in the three most salient measures: Time in Proximity to Stimulus Cage, Time Spent Exploring Stimulus Cage, and Time Spent Nose Touching. Looking at the exact same behavioral outcomes in Chapter 3, it was only the VIN-treated males who did not display a novelty preference. While A1221 appeared to disrupt the ability for both sexes to display a novelty preference in the 2-day dose paradigm, the 11-day dosed animals displayed the expected phenotype consistent with an ability to discriminate the two options. Similarly, the

significant lowering of male Nose Touching due to PCB treatment was unique to Chapter 2. As previously stated, it appears that differences in the dose- and/or behavioral-paradigms of Chapter 2 and 3 ameliorated the PCB-mediated disruption to Novelty Preference. It bears repeating that, in either of these two chapters (2 and 3) which utilizes Novelty Preference as a means of understanding the discriminatory abilities of the animals, caution must be taken when interpreting the meaning behind animals who failed to display the expected (novel) preference. In order to have a preference, an animal must first be able to discriminate between two options. Therefore, it can be stated with certainty that animals who showed this preference are, at least in part, unaffected in the processed that are involved in social recognition. Conversely, animals who fail to display a clear preference cannot be as-easily characterized. To say that these animals have an alteration in their ability to discriminate between the two options assumes that a preference must follow the ability to discriminate. Given the behavioral methods used in these chapters, such a distinction cannot be made with certainty.

**Simple Models of Social Behavior Should Not Be Used to Anticipate how An Animal Will Behave in More Complex Models of Social Interaction**

Aside from a small number of subtle effects of treatment observed in Chapter 4, the most interesting finding was that, when presented with four stimulus options (one option tantamount to a reproductively active member of the opposite sex), the preferences were not as clearly defined as they were in the binary models used in Chapters 2 and 3. This is in contrast to binary choice models of mate preference that indicate a significant preference for females to spend more time affiliating with a male castrate with testosterone replacement over a castrate without testosterone. The implications of this

finding suggest that caution must be made when extrapolating the biological implications of binary choice models.

### PCBS Given During Late Gestation Do Not Alter the Total Number of Oxytocin- or Vasopressin-Producing Neurons in the Paraventricular or Supraoptic Nuclei

Although the behaviors of these animals, as shown in Chapter 2, indicate that there was a sex- and dose-specific alteration of the novelty preference, there was no morphological indication that PCBs altered the amount of OT or AVP at the main sites of production within the hypothalamus. Looking at the rostral-to-caudal distribution of these OT/AVP-positive cells, however, there is an appreciable shift in the pattern. Using both count and density as a metric, the waxing and waning of the neurons throughout this region is not concordant between all treatment groups; this is especially apparent in A1221 (1.0) male OT distribution in the PVN and A1221 (0.5) female AVP distribution in the PVN. Though the relatively high degree of variability and low number of subjects (7-8 per group) it is possible that the subtle effects of PCBs were unable to be resolved by limited statistical power.

## IMPLICATIONS AND FUTURE DIRECTIONS

Though this dissertation has established that gestational exposes to EDCs can and do result in significant differences in the adult social behavior of animals, the mechanistic underpinnings of these observations remain unclear. Specifically, what serves as the motivational or perceptual antecedent in the groups that fail to display a novelty preference? In order to pursue further investigation of the proximate factors involved in

the altered phenotype, it is crucial to place the present findings within the context of what is known about how social information is processed in the brain.

Although the exhibition of a behavior cannot be attributed to any one part of the brain, there have been key distinct (but interconnected) regions identified in mammals that work together to create and regulate social behavior. Sex differences in behavior have also been attributed to sexually dimorphic features of these regions, quantified by differences in cell number, gene expression, or epigenetic characteristics. Furthermore, gonadal sex hormones produce a profound influence on the male- or female-typical behaviors in which these regions have been implicated.

The limbic system of the brain is predominately associated with the integration of external stimuli of social and/or sexual salience which results in the manifestation of appropriate behaviors that function to facilitate the protection of the individual and subsequent preservation of the species. Environmental sensory information is transmitted via the olfactory bulb and tract systems to the medial amygdala (MeA), which plays a critical role in the processing of the signals that trigger social behaviors. Although the exact mechanisms underlying these processes are not well understood, experiments in rats and mice have determined the importance of the MeA in affiliative and, more specifically, discriminatory capabilities of the animal within a social context. Exceptionally important in social recognition is the estrogen-dependent actions of oxytocin in this brain region. It is tempting to speculate that the hormone-sensitive organization of this region serves as the conduit by which the sexes were differentially altered by gestational EDCs. In light of no observable changes to the source of oxytocin in Chapter 5, the extrahypothalamic actions of this peptide remain uninvestigated. Moreover, projections from the MeA feed into the hypothalamic portion of the limbic system, also crucial in social behaviors.

Classically, the ventral medial nucleus of the hypothalamus (VMN) and medial preoptic area (mPOA) have been implicated in the female- or male-typical social and sexual behaviors, respectively. More recently, it has been proposed that both of these regions are of equal importance in the organization and activation of sex-specific behaviors. Rather than two separate neural circuits responsible for sexually dimorphic (but complimentary) copulatory behavior, attention has turned to a common network that, through sex differences in cell number, hormone and neuropeptide receptors, steroidogenic or epigenetic factors, result in the observed behavioral phenotypes. A growing body of evidence that EDCs can interfere with any one of these outcomes suggests that the sex-specific effects of gestational EDCs may be mediated by alterations to these processes. Present in the EDC literature and corroborated in this dissertation is the common occurrence of exacerbated sex differences; either induced or abolished. Future work focusing on these three brain regions in particular will provide crucial insight into how EDCs can alter the social information processing in a sexually dimorphic manner.

Ongoing work in the Gore lab will use the brains of the behaviorally characterized animals from Chapter 3 and employ the use of a TaqMan Low-Density qPCR Array (TLDA; Life Technologies) to explore the effects of EDCs in these three brain regions by determining the relative expression of 48 genes involved in hormone or neuropeptide signaling, steroidogenesis, epigenetic modifications, neurotransmission, and the expression of neurotrophic factors (Table 6.1). This will provide a unique and holistic perspective in how gestational exposure to PCBs or VIN may alter the behavioral phenotype. It will be interesting to see if changes in the medial amygdala, specifically in the genes involved in oxytocin or estrogen signaling, correlate with the loss of novelty preference in VIN males. The results from these experiments will also provide

information which may provide a reasoning behind the restoration of the expected novelty preference in the PCB-treated females (in contrast to the results of Chapter 2). It is not surprising that exposure to EDCs at different stages of gestation differentially alter the developmental trajectory in a manner that results in dissimilar behavioral outcomes. It will also be interesting to compare and contrast the effects of PCB and VIN treatment as determined by the TLDA study.

| Gene | | Gene Product | Function |
|---|---|---|---|
| 1 | Dnmt1 | DNA methyltransferase 1 | Epigenetics |
| 2 | Dmnt3a | DNA methyltransferase 3 alpha | Epigenetics |
| 3 | Dnmt3b | DNA methyltransferase 3 beta | Epigenetics |
| 4 | Hdac2 | Histone deacetylase 2 | Epigenetics |
| 5 | Hdac4 | Histone deacetylase 4 | Epigenetics |
| 6 | Esr1 | Estrogen receptor alpha | Hormone signaling |
| 7 | Esr2 | Estrogen receptor beta | Hormone signaling |
| 8 | Ar | Androgen receptor | Hormone signaling |
| 9 | Gper | GpProtein-coupled estrogen receptor | Hormone signaling |
| 10 | Pgr | Progesterone receptor | Hormone signaling |
| 11 | Nr3c1 | Glucocorticoid receptor | Hormone signaling |
| 12 | Esrra | Estrogen-related receptor alpha | Hormone signaling |
| 13 | Esrrb | Estrogen-related receptor beta | Hormone signaling |
| 14 | Esrrg | Estrogen-related receptor gamma | Hormone signaling |
| 15 | Crh | Corticotropin-releasing hormone | Hormone signaling |
| 16 | Crhr1 | Corticotropin-releasing hormone receptor 1 | Hormone signaling |
| 17 | Gnrh1 | Gonadotropin-releasing hormone | Hormone signaling |
| 18 | Per2 | Period circadian protein 2 | Hormone signaling |
| 19 | Oxt | Oxytocin | Neuropeptide Signaling |
| 20 | Oxtr | Oxytocin receptor | Neuropeptide Signaling |
| 21 | Avp | Argenine vasopressin | Neuropeptide Signaling |
| 22 | Avpr1a | Argenine vasopressin receptor subtype 1a | Neuropeptide Signaling |
| 23 | Kiss1 | Kisspeptin 1 | Neuropeptide Signaling |
| 24 | Kiss1r | Kisspeptin 1 receptor | Neuropeptide Signaling |
| 25 | Tac2 | Tachykinin 2 | Neuropeptide Signaling |
| 26 | Tac3 | Tachykinin 3 | Neuropeptide Signaling |
| 27 | Gabbr1 | GABA-B receptor 1 | Neurotransmission |
| 28 | Drd1 | Dopamine receptor D1 | Neurotransmission |
| 29 | Drd2 | Dopamine receptor D2 | Neurotransmission |
| 30 | Grin1 | NMDA receptor | Neurotransmission |
| 31 | Grin2b | NMDA receptor | Neurotransmission |
| 32 | Gad1 | Glutamate decarboxylase | Neurotransmission |
| 33 | Bdnf | Brain derived neurotrophic factor | Neurotrophic factor |
| 34 | Igf1 | Insulin-like growth factor 1 | Neurotrophic factor |
| 35 | Igf1r | Insulin-like growth factor 1 receptor | Neurotrophic factor |
| 36 | Cyp19a1 | Aromatase | Steroidogenesis |
| 37 | Srd5a1 | 5 alpha reductase 1 | Steroidogenesis |
| 38 | Hsd3b1 | 3 beta hydroxysteroid dehydrogenase 3b1 | Steroidogenesis |
| 39 | Star | Steroidogenic acute regulatory protein | Steroidogenesis |
| 40 | Cyp11a1 | Cytochrome P450 family 11 subfamily a | Steroidogenesis |
| 41 | Hsd17b1 | 17 beta hydroxysteroid dehydrogenase 1 | Steroidogenesis |
| 42 | Ahr | Arylhydrocarbon receptor | Transcription factor |
| 43 | Egr1 | Early growth response 1 | Transcription factor |
| 44 | Foxp1 | Forkhead box protein 1 | Transcription factor |
| 45 | Foxp2 | Forkhead box protein 2 | Transcription factor |
| 46 | Arntl | Aryl hydrocarbon receptor nuclear translocator-like protein | Transcription factor |
| 47 | Gapdh/Rpl1 | Glyceraldehyde-3- Phosphate Dehydrogenase | Housekeeping |
| 48 | 18s | 18S ribosomal RNA | Housekeeping |

Table 6.1. TLDA Genes

A list of the 48 genes comprising the custom-designed microfluidic TaqMan Low Density Array with the gene product and general role indicated.

## CLOSING REMARKS

The use of behavior as a tool has proven an invaluable means of identifying the biological significance of environmental contamination. This dissertation adds to a growing body of literature that exposure to EDCs during critical periods of development forever alter the developmental trajectory of the individual. While we have established the behavioral implications of such interference, further experimentation on the mechanisms and brain regions underlying the manifestations of these sexually dimorphic behaviors will provide much-needed insight into the nature of this disruption. Determining the mechanisms by which past-and-presently known EDCs interfere with homeostasis can and should be used to inform the identification of other potential EDCs that have contaminated the environment. Moving into the future, there is no reason to believe that the production and subsequent environmental release of novel synthetic chemicals will be halted. Being able to establish and quantify known EDCs and their actions may lead to more stringent regulations that may identify and condemn particularly harmful compounds before, rather than after, their widespread use.

# Bibliography


Acher, R., & Chauvet, J. (1995). The neurohypophysial endocrine regulatory cascade: precursors, mediators, receptors, and effectors. *Frontiers in Neuroendocrinology*, *16*, 237–289. https://doi.org/10.1006/frne.1995.1009

Aiello, T. P., & Whitaker-Azmitia, P. M. (2011). Sexual differentiation and the neuroendocrine hypothesis of autism. *Anatomical Record (Hoboken, N.J. : 2007)*, *294*(10), 1663–70. https://doi.org/10.1002/ar.21251

Altstein, M., Whitnall, M. H., House, S., Key, S., & Gainer, H. (1988). An immunochemical analysis of oxytocin and vasopressin prohormone processing in vivo. *Peptides*, *9*(1), 87–105. https://doi.org/10.1016/0196-9781(88)90014-9

Alves, S. E., Lopez, V., McEwen, B. S., & Weiland, N. G. (1998). Differential colocalization of estrogen receptor beta (ERbeta) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: an immunocytochemical study. *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 3281–3286. https://doi.org/10.1073/pnas.95.6.3281

Andrews, O., Buffington, S. A., Viana, G., Prisco, D., Auchtung, T. A., Ajami, N. J., … Sakuma, Y. (2003). Sex-specific effects of gonadal steroids on conspecific odor preference in the rat. *Hormones and Behavior*, *466*(August), 356–361. https://doi.org/10.1016/j.yhbeh.2004.05.008

Arcaro, K. F., Yi, L., Seegal, R. F., Vakharia, D. D., Yang, Y., Spink, D. C., … Gierthy, J. F. (1999). 2,2′,6,6′-Tetrachlorobiphenyl is estrogenic in vitro and in vivo. *Journal of Cellular Biochemistry*, *72*(1), 94–102. https://doi.org/10.1002/(SICI)1097-4644(19990101)72:1<94::AID-JCB10>3.0.CO;2-Y

Arnold, A. P. (2009). The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. *Hormones and Behavior*, *55*, 570–578. https://doi.org/10.1016/j.yhbeh.2009.03.011

Arnold, A. P., & Breedlove, S. M. (1985). Organizational and activational effects of sex steroids on brain and behavior: A reanalysis. *Hormones and Behavior*, *19*(4), 469–498. https://doi.org/10.1016/0018-506X(85)90042-X

Aron, C. (1979). Mechanisms of Control of the Reproductive Function by Olfactory Stimuli in Female Mammals. *Physiological Reviews*, *52*(2), 229–284.

Bakker, J. (2003). Sexual differentiation of the neuroendocrine mechanisms regulating mate recognition in mammals. *Journal of Neuroendocrinology*, *15*(6), 615–621. https://doi.org/10.1046/j.1365-2826.2003.01099.x

Bale, T. L., Davis, A. M., Auger, A. P., Dorsa, D. M., & McCarthy, M. M. (2001). CNS region-specific oxytocin receptor expression: importance in regulation of anxiety and sex behavior. *The Journal of Neuroscience*, *21*(7), 2546–52. https://doi.org/21/7/2546 [pii]

Bellingham, M., Fiandanese, N., Byers, A., Cotinot, C., Evans, N. P., Pocar, P., … Fowler, P. a. (2012). Effects of exposure to environmental chemicals during pregnancy on the development of the male and female reproductive axes. *Reproduction in Domestic Animals = Zuchthygiene*, *47 Suppl 4*, 15–22. https://doi.org/10.1111/j.1439-0531.2012.02050.x

Belloni, V., Dessì-Fulgheri, F., Zaccaroni, M., Di Consiglio, E., De Angelis, G., Testai, E., … Santucci, D. (2011). Early exposure to low doses of atrazine affects behavior in juvenile and adult CD1 mice. *Toxicology*, *279*, 19–26. https://doi.org/10.1016/j.tox.2010.07.002

Bergvall, Å. H., Vega Matuszczyk, J., Dahlöf, L. G., & Hansen, S. (1991). Peripheral anosmia attenuates female-enhanced aggression in male rats. *Physiology and Behavior*, *50*(1), 33–40. https://doi.org/10.1016/0031-9384(91)90494-9

Betz, A. J., Jayatilaka, S., Joshi, J., Ramanan, S., Debartolo, D., Pylypiw, H., & Franke, E. (2013). Chronic exposure to benzyl butyl phthalate (BBP) alters social interaction and fear conditioning in male adult rats: Alterations in amygdalar MeCP2, ERK1/2 and ERα. *Neuroendocrinology Letters*, *34*(5), 347–358.

Bielsky, I. F., Hu, S.-B., Szegda, K. L., Westphal, H., & Young, L. J. (2004). Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *29*, 483–493. https://doi.org/10.1038/sj.npp.1300360

Bielsky, I. F., Hu, S. B., Ren, X., Terwilliger, E. F., & Young, L. J. (2005). The V1a vasopressin receptor is necessary and sufficient for normal social recognition: A gene replacement study. *Neuron*, *47*, 503–513. https://doi.org/10.1016/j.neuron.2005.06.031

Boer, G. J., Gash, D. M., Dick, L., & Schluter, N. (1985). Vasopressin neuron survival in neonatal brattleboro rats; critical factors in graft development and innervation of the host brain. *Neuroscience*, *15*, 1087–1109. https://doi.org/10.1016/0306-4522(85)90255-6

Böttner, M., & Wuttke, W. (2005). Chronic treatment with low doses of estradiol affects pituitary and thyroid function in young and middle-aged ovariectomized rats. *Biogerontology*, *6*(4), 261–9. https://doi.org/10.1007/s10522-005-2623-2

Buckley, J., Willingham, E., Agras, K., & Baskin, L. S. (2006). Embryonic exposure to the fungicide vinclozolin causes virilization of females and alteration of progesterone receptor expression in vivo: An experimental study in mice. *Environmental Health: A Global Access Science Source*, *5*. https://doi.org/10.1186/1476-069X-5-4

Cabras, P., & Angioni, A. (2000). Pesticide residues in grapes, wine, and their processing products. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf990727a

Cao, J., Rebuli, M. E., Rogers, J., Todd, K. L., Leyrer, S. M., Ferguson, S. a, & Patisaul, H. B. (2013). Prenatal bisphenol a exposure alters sex-specific estrogen receptor expression in the neonatal rat hypothalamus and amygdala. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, *133*(1), 157–73. https://doi.org/10.1093/toxsci/kft035

Carrier, N., & Kabbaj, M. (2012). Sex differences in social interaction behaviors in rats are mediated by extracellular signal-regulated kinase 2 expression in the medial prefrontal cortex. *Neuroscience*, *212*, 86–92. https://doi.org/10.1016/j.neuroscience.2012.03.041

Carson, H. L. (2003). Mate choice theory and the mode of selection in sexual populations. *Pnas*, *100*(11), 6584–6587. https://doi.org/10.1073/pnas.0732174100

Carter, C. S., Boone, E. M., Pournajafi-Nazarloo, H., & Bales, K. L. (2009). Consequences of early experiences and exposure to oxytocin and vasopressin are sexually dimorphic. *Developmental Neuroscience*. https://doi.org/10.1159/000216544

Choleris, E., Gustafsson, J.-A., Korach, K. S., Muglia, L. J., Pfaff, D. W., & Ogawa, S. (2003). An estrogen-dependent four-gene micronet regulating social recognition: A study with oxytocin and estrogen receptor- and - knockout mice. *Proceedings of the National Academy of Sciences*, *100*(10), 6192–6197. https://doi.org/10.1073/pnas.0631699100

Choleris, E., Ogawa, S., Kavaliers, M., Gustafsson, J. Å., Korach, K. S., Muglia, L. J., & Pfaff, D. W. (2006). Involvement of estrogen receptor α, β and oxytocin in social discrimination: A detailed behavioral analysis with knockout female mice. *Genes, Brain and Behavior*, *5*, 528–539. https://doi.org/10.1111/j.1601-183X.2006.00203.x

Choleris, E., Ogawa, S., Kavaliers, M., Gustafsson, J. Å., Korach, K. S., Muglia, L. J., & Pfaff, D. W. (2006). Involvement of estrogen receptor α, β and oxytocin in social discrimination: A detailed behavioral analysis with knockout female mice. *Genes, Brain and Behavior*, *5*, 528–539. https://doi.org/10.1111/j.1601-183X.2006.00203.x

Cooke, B. M., Tabibnia, G., & Breedlove, S. M. (1999). A brain sexual dimorphism controlled by adult circulating androgens. *Proceedings of the National Academy of Sciences*, *96*(13), 7538–7540. https://doi.org/10.1073/pnas.96.13.7538

Cox, K. H., & Rissman, E. F. (2011). Sex differences in juvenile mouse social behavior are influenced by sex chromosomes and social context. *Genes, Brain and Behavior*, *10*, 465–472. https://doi.org/10.1111/j.1601-183X.2011.00688.x

Crews, D., Gillette, R., Scarpino, S. V., Manikkam, M., Savenkova, M. I., & Skinner, M. K. (2012). Epigenetic transgenerational inheritance of altered stress responses. *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.1118514109

Crews, D., & Gore, A. C. (2011). Life imprints: Living in a contaminated world. *Environmental Health Perspectives*. https://doi.org/10.1289/ehp.1103451

Crews, D., Lou, W., Fleming, A., & Ogawa, S. (2006). From gene networks underlying sex determination and gonadal differentiation to the development of neural networks regulating sociosexual behavior. *Brain Research*, *1126*(1), 109–121. https://doi.org/10.1016/j.brainres.2006.07.031

Cromwell, H. C., Johnson, A., McKnight, L., Horinek, M., Asbrock, C., Burt, S., … Meserve, L. A. (2007). Effects of polychlorinated biphenyls on maternal odor conditioning in rat pups. *Physiology and Behavior*, *91*(5), 658–666. https://doi.org/10.1016/j.physbeh.2007.03.029

Davis, E. C., Popper, P., & Gorski, R. A. (1996). The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area. *Brain Research*, *734*, 10–18.

Davis, E. C., Shryne, J. E., & Gorski, R. A. (1996). Structural sexual dimorphisms in the anteroventral periventricular nucleus of the rat hypothalamus are sensitive to gonadal steroids perinatally, but develop peripubertally. *Neuroendocrinology*, *63*(2), 142–148. https://doi.org/10.1159/000126950

De Jesús-Burgos, M., Torres-Llenza, V., & Pérez-Acevedo, N. L. (2012). Activation of amygdalar metabotropic glutamate receptors modulates anxiety, and risk assessment behaviors in ovariectomized estradiol-treated female rats. *Pharmacology, Biochemistry, and Behavior*, *101*(3), 369–78. https://doi.org/10.1016/j.pbb.2012.01.016

de Vries, G. J., Duetz, W., Buijs, R. M., van Heerikhuize, J., & Vreeburg, J. T. (1986). Effects of androgens and estrogens on the vasopressin and oxytocin innervation of the adult rat brain. *Brain Research*, *399*, 296–302. https://doi.org/10.1016/0006-8993(86)91519-2

De Vries, G. J., & Al-Shamma, H. A. (1990). Sex differences in hormonal responses of vasopressin pathways in the rat brain. *Journal of Neurobiology*, *21*, 686–693. https://doi.org/10.1002/neu.480210503

de Vries, G. J. (2008). Sex differences in vasopressin and oxytocin innervation of the brain. *Progress in Brain Research*. https://doi.org/10.1016/S0079-6123(08)00402-0

DeKoning, E. P., & Karmaus, W. (2000). PCB exposure in utero and via breast milk. A review. *Journal of Exposure Analysis and Environmental Epidemiology*, *10*(3), 285–93. https://doi.org/10.1038/sj.jea.7500090

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., … Gore, A. C. (2009). Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. *Endocrine Reviews*, *30*(4), 293–342. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2726844&tool=pmcentrez&rendertype=abstract

Dickerson, S. M., Cunningham, S. L., & Gore, A. C. (2011). Prenatal PCBs disrupt early neuroendocrine development of the rat hypothalamus. *Toxicology and Applied Pharmacology*, *252*, 36–46. https://doi.org/10.1016/j.taap.2011.01.012

Dickerson, S. M., Cunningham, S. L., Patisaul, H. B., Woller, M. J., & Gore, A. C. (2011). Endocrine disruption of brain sexual differentiation by developmental PCB exposure. *Endocrinology*, *152*, 581–594. https://doi.org/10.1210/en.2010-1103

Dickerson, S. M., & Gore, A. C. (2007). Estrogenic environmental endocrine-disrupting chemical effects on reproductive neuroendocrine function and dysfunction across the life cycle. *Reviews in Endocrine and Metabolic Disorders*. https://doi.org/10.1007/s11154-007-9048-y

Dickerson, S. M., Guevara, E., Woller, M. J., & Gore, A. C. (2009). Cell death mechanisms in GT1-7 GnRH cells exposed to polychlorinated biphenyls PCB74, PCB118, and PCB153. *Toxicology and Applied Pharmacology*, *237*, 237–245. https://doi.org/10.1016/j.taap.2009.04.001

Donaldson, Z. R., & Young, L. J. (2008). Oxytocin, vasopressin, and the neurogenetics of sociality. *Science (New York, N.Y.)*, *322*, 900–904. https://doi.org/10.1126/science.1158668

Ebstein, R. P., Israel, S., Lerer, E., Uzefovsky, F., Shalev, I., Gritsenko, I., … Yirmiya, N. (2009). Arginine vasopressin and oxytocin modulate human social behavior. In *Annals of the New York Academy of Sciences* (Vol. 1167, pp. 87–102). https://doi.org/10.1111/j.1749-6632.2009.04541.x

Egashira, N., Tanoue, A., Matsuda, T., Koushi, E., Harada, S., Takano, Y., … Fujiwara, M. (2007). Impaired social interaction and reduced anxiety-related behavior in vasopressin V1a receptor knockout mice. *Behavioural Brain Research*, *178*(1), 123–127. https://doi.org/10.1016/j.bbr.2006.12.009

Engelmann, M., Wotjak, C. T., & Landgraf, R. (1995). Social discrimination procedure: An alternative method to investigate juvenile recognition abilities in rats. *Physiology and Behavior*, *58*, 315–321. https://doi.org/10.1016/0031-9384(95)00053-L

Farabollini, F., Porrini, S., Della Seta, D., Bianchi, F., & Dessì-Fulgheri, F. (2002). Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. *Environmental Health Perspectives*, *110*(SUPPL. 3), 409–414. https://doi.org/10.1289/ehp.02110s3409

Ferguson, J. N., Aldag, J. M., Insel, T. R., & Young, L. J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(20), 8278–85. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11588199

Ferreira-Nuño, A., Morales-Otal, A., Paredes, R. G., & Velázquez-Moctezuma, J. (2005). Sexual behavior of female rats in a multiple-partner preference test. *Hormones and Behavior*, *47*(3), 290–296. https://doi.org/10.1016/j.yhbeh.2004.11.012

Frame, G. M., Wagner, R. E., Carnahan, J. C., Brown, J. F., May, R. J., Smullen, L. A., & Bedard, D. L. (1996). ON DB-I CAPILLARY GC COLUMNS, *33*(4), 603–623.

Frye, C., Bo, E., Calamandrei, G., Calzà, L., Dessì-Fulgheri, F., Fernández, M., … Panzica, G. C. (2012). Endocrine disrupters: A review of some sources, effects, and mechanisms of actions on behaviour and neuroendocrine systems. *Journal of Neuroendocrinology*. https://doi.org/10.1111/j.1365-2826.2011.02229.x

Frye, C. A. (2014). Endocrine-Disrupting Chemicals. Elucidating Our Understanding of Their Role in Sex and Gender-Relevant End Points. *Vitamins and Hormones*, *94*, 41–98. https://doi.org/10.1016/B978-0-12-800095-3.00003-1

Giesy, J., & Kannan, K. (1998). Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. *Crit Rev Toxicol*, *28*(6), 511–569.

Gillette, R., Miller-Crews, I., Nilsson, E. E., Skinner, M. K., Gore, A. C., & Crews, D. (2014). Sexually dimorphic effects of ancestral exposure to vinclozolin on stress reactivity in rats. *Endocrinology*, en20141253. https://doi.org/10.1210/en.2014-1253

Gillette, R., Miller-Crews, I., Nilsson, E. E., Skinner, M. K., Gore, A. C., & Crews, D. (2014). Sexually dimorphic effects of ancestral exposure to vinclozolin on stress reactivity in rats. *Endocrinology*, *155*(10), 3853–3866. https://doi.org/10.1210/en.2014-1253

Gillette, R., Reilly, M. P., Topper, V. Y., Thompson, L. M., Crews, D., & Gore, A. C. (2017). Anxiety-like behaviors in adulthood are altered in male but not female rats exposed to low dosages of polychlorinated biphenyls in utero. *Hormones and Behavior*, *87*, 8–15. https://doi.org/10.1016/j.yhbeh.2016.10.011

Gladen, B. C., Doucet, J., & Hansen, L. G. (2003). Assessing human polychlorinated biphenyl contamination for epidemiologic studies: Lessons from patterns of Congener concentrations in Canadians in 1992. *Environmental Health Perspectives*, *111*(4), 437–443. https://doi.org/10.1289/ehp.5858

Gore, A. C., Chappell, V. A., Fenton, S. E., Flaws, J. A., Nadal, A., Prins, G. S., … Zoeller, R. T. (2015). Executive Summary to EDC-2: The Endocrine Society's second Scientific Statement on endocrine-disrupting chemicals. *Endocrine Reviews*. https://doi.org/10.1210/er.2015-1093

Gorski, R. A., Harlan, R. E., Jacobson, C. D., Shryne, J. E., & Southam, A. M. (1980). Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat. *The Journal of Comparative Neurology*, *193*(2), 529–39. https://doi.org/10.1002/cne.901930214

Grandjean, P., Barouki, R., Bellinger, D. C., Casteleyn, L., Chadwick, L. H., Cordier, S., … Heindel, J. J. (2015). Life-long implications of developmental exposure to environmental stressors: New perspectives. *Endocrinology*, *156*(10), 3408–3415. https://doi.org/10.1210/EN.2015-1350

Gréco, B., Allegretto, E. A., Tetel, M. J., & Blaustein, J. D. (2001). Coexpression of ER beta with ER alpha and progestin receptor proteins in the female rat forebrain: effects of estradiol treatment. *Endocrinology*, *142*, 5172–5181. https://doi.org/10.1210/en.142.12.5172

Henley, C. L., Nunez, A. A., & Clemens, L. G. (2011). Hormones of choice: The neuroendocrinology of partner preference in animals. *Frontiers in Neuroendocrinology*. https://doi.org/10.1016/j.yfrne.2011.02.010

Hines, M., Allen, L. S., & Gorski, R. A. (1992). Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat. *Brain Research*, *579*(2), 321–326. https://doi.org/10.1016/0006-8993(92)90068-K

Hong, W., Kennedy, A., Burgos-Artizzu, X. P., Zelikowsky, M., Navonne, S. G., Perona, P., & Anderson, D. J. (2015). Automated measurement of mouse social behaviors using depth sensing, video tracking, and machine learning. *Proceedings of the National Academy of Sciences*, *112*(38), E5351–E5360. https://doi.org/10.1073/pnas.1515982112

Hopf, N. B., Ruder, A. M., & Succop, P. (2009). Background levels of polychlorinated biphenyls in the U.S. population. *The Science of the Total Environment*, *407*(24), 6109–19. https://doi.org/10.1016/j.scitotenv.2009.08.035

Ito, S., Murakami, S., Yamanouchi, K., & Arai, Y. (1986). Prenatal androgen exposure, preoptic area and reproductive functions in the female rat. *Brain and Development*, *8*(4), 463–468. https://doi.org/10.1016/S0387-7604(86)80070-5

Jacobsen, P. R., Axelstad, M., Boberg, J., Isling, L. K., Christiansen, S., Mandrup, K. R., … Hass, U. (2012). Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reproductive Toxicology*, *34*, 237–250. https://doi.org/10.1016/j.reprotox.2012.05.099

Jacobson, C. D., Shryne, J. E., Shapiro, F., & Gorski, R. A. (1980). Ontogeny of the sexually dimorphic nucleus of the preoptic area. *The Journal of Comparative Neurology*, *193*, 541–548. https://doi.org/10.1002/cne.901930215

Jessen, H. M., Kolodkin, M. H., Bychowski, M. E., Auger, C. J., & Auger, A. P. (2010). The nuclear receptor corepressor has organizational effects within the developing amygdala on juvenile social play and anxiety-like behavior. *Endocrinology*, *151*(3), 1212–20. https://doi.org/10.1210/en.2009-0594

Johnston, R. E. (2003). CHEMICAL COMMUNICATION IN RODENTS: FROM PHEROMONES TO INDIVIDUAL RECOGNITION. *Journal of Mammalogy*. https://doi.org/10.1644/BLe-010

Jolous-Jamshidi, B., Cromwell, H. C., McFarland, A. M., & Meserve, L. A. (2010). Perinatal exposure to polychlorinated biphenyls alters social behaviors in rats. *Toxicology Letters*, *199*, 136–143. https://doi.org/10.1016/j.toxlet.2010.08.015

Jolous-Jamshidi, B., Cromwell, H. C., McFarland, A. M., & Meserve, L. A. (2010). Perinatal exposure to polychlorinated biphenyls alters social behaviors in rats. *Toxicology Letters*, *199*, 136–143. https://doi.org/10.1016/j.toxlet.2010.08.015

Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., & Woodgett, J. R. (2011). Assessment of social interaction behaviors. *Journal of Visualized Experiments : JoVE*, (48), 6–10. https://doi.org/10.3791/2473

Kandaraki, E., Chatzigeorgiou, A., Livadas, S., Palioura, E., Economou, F., Koutsilieris, M., … Diamanti-Kandarakis, E. (2011). Endocrine Disruptors and Polycystic Ovary Syndrome (PCOS): Elevated Serum Levels of Bisphenol A in Women with PCOS. *The Journal of Clinical Endocrinology & Metabolism*, *96*(3), E480–E484. https://doi.org/10.1210/jc.2010-1658

Kawaguchi, M., Morohoshi, K., Imai, H., Morita, M., Kato, N., & Himi, T. (2010). Maternal exposure to isobutyl-paraben impairs social recognition in adult female rats. *Experimental Animals / Japanese Association for Laboratory Animal Science*, *59*(5), 631–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21030791

Keverne, E. B., & Curley, J. P. (2004). Vasopressin, oxytocin and social behaviour. *Current Opinion in Neurobiology*. https://doi.org/10.1016/j.conb.2004.10.006

Kim, K. C., Kim, P., Go, H. S., Choi, C. S., Yang, S.-I., Cheong, J. H., … Ko, K. H. (2011). The critical period of valproate exposure to induce autistic symptoms in Sprague-Dawley rats. *Toxicology Letters*, *201*(2), 137–42. https://doi.org/10.1016/j.toxlet.2010.12.018

Kipervarg, Y. (1992). Stress: Neurobiology and Neuroendocrinology. *The Yale Journal of Biology and Medicine*, *65*(1), 56–57.

Kundakovic, M., Gudsnuk, K., Franks, B., Madrid, J., Miller, R. L., Perera, F. P., & Champagne, F. a. (2013). Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 9956–61. https://doi.org/10.1073/pnas.1214056110

Kuwahara, R., Kawaguchi, S., Kohara, Y., Cui, H., & Yamashita, K. (2013). Perinatal Exposure to Low-Dose Bisphenol A Impairs Spatial Learning and Memory in Male Rats. *Journal of Pharmacological Sciences*, *123*, 132–139. https://doi.org/10.1254/jphs.13093FP

Laflamme, N., Nappi, R. E., Drolet, G., Labrie, C., & Rivest, S. (1998). Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. *Journal of Neurobiology*, *36*, 357–378.

Landgraf, R., & Neumann, I. D. (2004). Vasopressin and oxytocin release within the brain: A dynamic concept of multiple and variable modes of neuropeptide communication. *Frontiers in Neuroendocrinology*. https://doi.org/10.1016/j.yfrne.2004.05.001

Lee, H., Yamanouchi, K., & Nishihara, M. (206AD). Effects of perinatal exposure to phthalate/adipate esters on hypothalamic gene expression and sexual behavior in rats. *J Reprod Dev*, *52*(3), 343–352.

Lee, H. J., Caldwell, H. K., Macbeth, A. H., Tolu, S. G., & Young, W. S. (2008). A conditional knockout mouse line of the oxytocin receptor. *Endocrinology*, *149*, 3256–3263. https://doi.org/10.1210/en.2007-1710

Leng, G., Brown, C. H., & Russell, J. A. (1999). Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Progress in Neurobiology*. https://doi.org/10.1016/S0301-0082(98)00072-0

Levine, S. (2001). Primary social relationships influence the development of the hypothalamic--pituitary--adrenal axis in the rat. *Physiology & Behavior*, *73*(3), 255–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11438350

Lin, Y., Pessah, I. N., & Puschner, B. (2013). Simultaneous determination of polybrominated diphenyl ethers and polychlorinated biphenyls by gas chromatography-tandem mass spectrometry in human serum and plasma. *Talanta*, *113*, 41–8. https://doi.org/10.1016/j.talanta.2013.04.001

Ludwig, M. (1995). Functional role of intrahypothalamic release of oxytocin and vasopressin: consequences and controversies. *The American Journal of Physiology*, *268*, E537–E545.

Margaret R. Bell, Lindsay M. Thompson, Karla Rodriguez, A. C. G. (2015). Two-hit exposure to polychlorinated biphenyls at gestational and juvenile life stages: 1. Sexually dimorphic effects on social and anxiety-like behaviors Margaret. *Hormones and Behavior*, *78*, 168–177. https://doi.org/10.1177/0963721412473755.Surging

Meaney, M. J., & Stewart, J. (1979). Environmental factors influencing the affiliative behavior of male and female rats (Rattus norvegicus). *Animal Learning & Behavior*. https://doi.org/10.3758/BF03209692

Milenkovic, M., Diaz-Garcia, C., Wallin, A., & Brännström, M. (2012). Viability and function of the cryopreserved whole rat ovary: comparison between slow-freezing and vitrification. *Fertility and Sterility*, 1–7. https://doi.org/10.1016/j.fertnstert.2012.01.123

Miller, M. A., Urban, J. H., & Dorsa, D. M. (1989). Steroid dependency of vasopressin neurons in the bed nucleus of the stria terminalis by in situ hybridization. *Endocrinology*, *125*, 2335–2340. https://doi.org/10.1210/endo-125-5-2335

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., … Crawley, J. N. (2004). Sociability and preference for social novelty in five inbred strains: An approach to assess autistic-like behavior in mice. *Genes, Brain and Behavior*, *3*, 287–302. https://doi.org/10.1111/j.1601-1848.2004.00076.x

Murakami, G., Hunter, R. G., Fontaine, C., Ribeiro, a, & Pfaff, D. (2011). Relationships among estrogen receptor, oxytocin and vasopressin gene expression and social interaction in male mice. *The European Journal of Neuroscience*, *34*(3), 469–77. https://doi.org/10.1111/j.1460-9568.2011.07761.x

Nadler, J. J., Moy, S. S., Dold, G., Trang, D., Simmons, N., Perez, A., … Crawley, J. N. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes, Brain and Behavior*, *3*, 303–314. https://doi.org/10.1111/j.1601-183X.2004.00071.x

Nishizuka, M., & Arai, D. Y. (1983). Male-female differences in the intra-amygdaloid input to the medial amygdala. *Experimental Brain Research*, *52*(3), 328–332. https://doi.org/10.1007/BF00238027

Noonan, L. R., Continella, G., & Pedersen, C. A. (1989). Neonatal administration of oxytocin increases novelty-induced grooming in the adult rat. *Pharmacology, Biochemistry, and Behavior*, *33*, 555–558. https://doi.org/10.1016/0091-3057(89)90386-9

Norman W. Heimstra, A. L. M. (1962). Social Influence on the Reponse to Drugs: IV. Stimulus Factors. *The Psychological Record*, *12*, 383–386.

Ostby, J., Monosson, E., & Gray, L. E. (1999). Environmental antiandrogens: Low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicology and Industrial Health*, *15*(2), 48–64. https://doi.org/10.1177/074823379901500106

Padmanabhan, V., Cardoso, R. C., & Puttabyatappa, M. (2016). Developmental programming, a pathway to disease. *Endocrinology*. https://doi.org/10.1210/en.2016-1003

Paredes, R. G., Tzschentke, T., & Nakach, N. (1998). Lesions of the medial preoptic area/anterior hypothalamus (MPOA/AH) modify partner preference in male rats. *Brain Research*, *813*(1), 1–8. https://doi.org/10.1016/S0006-8993(98)00914-7

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*, e45. https://doi.org/10.1093/nar/29.9.e45

Phoenix, C. H., Goy, R. W., Gerall, A. A., & Young, W. C. (1959). ORGANIZING ACTION OF PRENATALLY ADMINISTERED TESTOSTERONE PROPIONATE ON THE TISSUES MEDIATING MATING BEHAVIOR IN THE FEMALE GUINEA PIG, *65*(3), 369–382.

Picton, H. M., Kim, S. S., & Gosden, R. G. (2000). Cryopreservation of gonadal tissue and cells. *British Medical Bulletin*, *56*(3), 603–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11255548

Pierman, S., Sica, M., Allieri, F., Viglietti-Panzica, C., Panzica, G. C., & Bakker, J. (2008). Activational effects of estradiol and dihydrotestosterone on social recognition and the arginine-vasopressin immunoreactive system in male mice lacking a functional aromatase gene. *Hormones and Behavior*, *54*(1), 98–106. https://doi.org/10.1016/j.yhbeh.2008.02.001

Platt, J. R. (1964). Strong Inference, *146*(3642), 347–353.

Porrini, S., Belloni, V., Seta, D. Della, Farabollini, F., Giannelli, G., & Dessì-Fulgheri, F. (2005). Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. In *Brain Research Bulletin* (Vol. 65, pp. 261–266). https://doi.org/10.1016/j.brainresbull.2004.11.014

Poster, P.-, & Lyon, C. B. (n.d.). ART , laboratory : cryopreservation of gonads P-404 Poster ART , laboratory : embryo selection. *Perfusion*, (July 2007), 157–158.

Reilly, M. P., Weeks, C. D., Topper, V. Y., Thompson, L. M., Crews, D., & Gore, A. C. (2015). The effects of prenatal PCBs on adult social behavior in rats. *Hormones and Behavior*, *73*(3), 47–55. https://doi.org/10.1016/j.yhbeh.2015.06.002

Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., & Prossnitz, E. R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science (New York, N.Y.)*, *307*, 1625–1630. https://doi.org/10.1126/science.1106943

Roselli, C. E., Horton, L. E., & Resko, J. A. (1985). Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology*, *117*(6), 2471–2477. https://doi.org/10.1210/endo-117-6-2471

Roselli, C. E., Larkin, K., Schrunk, J. M., & Stormshak, F. (2004). Sexual partner preference, hypothalamic morphology and aromatase in rams. *Physiology and Behavior*. https://doi.org/10.1016/j.physbeh.2004.08.017

Ross, H. E., & Young, L. J. (2009). Oxytocin and the neural mechanisms regulating social cognition and affiliative behavior. *Frontiers in Neuroendocrinology*. https://doi.org/10.1016/j.yfrne.2009.05.004

Sakamoto, H., Matsuda, K. I., Hosokawa, K., Nishi, M., Morris, J. F., Prossnitz, E. R., & Kawata, M. (2007). Expression of G protein-coupled receptor-30, a G protein-coupled membrane estrogen receptor, in oxytocin neurons of the rat paraventricular and supraoptic nuclei. *Endocrinology*, *148*, 5842–5850. https://doi.org/10.1210/en.2007-0436

Sawchenko, P. E. (1987). Evidence for differential regulation of corticotropin-releasing factor and vasopressin immunoreactivities in parvocellular neurosecretory and autonomic-related projections of the paraventricular nucleus. *Brain Research*, *437*(2), 253–263. https://doi.org/10.1016/0006-8993(87)91641-6

Schug, T. T., Janesick, A., Blumberg, B., & Heindel, J. J. (2011). Endocrine disrupting chemicals and disease susceptibility. *Journal of Steroid Biochemistry and Molecular Biology*. https://doi.org/10.1016/j.jsbmb.2011.08.007

Scordalakes, E. M., & Rissman, E. F. (2004). Aggression and arginine vasopressin immunoreactivity regulation by androgen receptor and estrogen receptor α. *Genes, Brain and Behavior*, *3*(1), 20–26. https://doi.org/10.1111/j.1601-183X.2004.00036.x

Shapiro, R. A., Xu, C., & Dorsa, D. M. (2000). Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor alpha and beta. *Endocrinology*, *141*, 4056–4064.

Shekhar, P. V. M., Werdell, J., & Basrur, V. S. (1997). Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. *Journal of the National Cancer Institute*, *89*(23), 1774–1782. https://doi.org/10.1093/jnci/89.23.1774

Shughrue, P. J., Lane, M. V, & Merchenthaler, I. (1997). Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *The Journal of Comparative Neurology*, *388*, 507–525. https://doi.org/10.1002/(SICI)1096-9861(19971201)388:4<507::AID-CNE1>3.0.CO;2-6 [pii]

Siviy, S. M., & Panksepp, J. (2011). In search of the neurobiological substrates for social playfulness in mammalian brains. *Neuroscience and Biobehavioral Reviews*, *35*(9), 1821–30. https://doi.org/10.1016/j.neubiorev.2011.03.006

Slamberová, R., Mikulecká, A., Pometlová, M., Schutová, B., Hrubá, L., & Deykun, K. (2011). Sex differences in social interaction of methamphetamine-treated rats. *Behavioural Pharmacology*, *22*, 617–623. https://doi.org/10.1097/FBP.0b013e32834afea4

So, N., Franks, B., Lim, S., & Curley, J. P. (2015). Associations between Mouse Social Dominance and Brain Gene Expression, 1–27. https://doi.org/10.1371/journal.pone.0134509

Sobolewski, M., Conrad, K., Allen, J. L., Weston, H., Martin, K., Lawrence, B. P., & Cory-Slechta, D. a. (2014). Sex-specific enhanced behavioral toxicity induced by maternal exposure to a mixture of low dose endocrine-disrupting chemicals. *Neurotoxicology*, *45C*, 121–130. https://doi.org/10.1016/j.neuro.2014.09.008

Stack, A., Carrier, N., Dietz, D., Hollis, F., Sorenson, J., & Kabbaj, M. (2010). Sex differences in social interaction in rats: role of the immediate-early gene zif268. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *35*, 570–580. https://doi.org/10.1038/npp.2009.163

Steinberg, R. M., Walker, D. M., Juenger, T. E., Woller, M. J., & Gore, A. C. (2008). Effects of perinatal polychlorinated biphenyls on adult female rat reproduction: development, reproductive physiology, and second generational effects. *Biology of Reproduction*, *78*, 1091–1101. https://doi.org/10.1095/biolreprod.107.067249

Steinberg, R. M., Juenger, T. E., & Gore, A. C. (2007). The effects of prenatal PCBs on adult female paced mating reproductive behaviors in rats. *Hormones and Behavior*, *51*, 364–372. https://doi.org/10.1016/j.yhbeh.2006.12.004

Stewart, P. W., Lonky, E., Reihman, J., Pagano, J., Gump, B. B., & Darvill, T. (2008). The relationship between prenatal PCB exposure and intelligence (IQ) in 9-year-old children. *Environmental Health Perspectives*, *116*(10), 1416–1422. https://doi.org/10.1289/ehp.11058

Sullivan, A. W., Beach, E. C., Stetzik, L. A., Perry, A., D'Addezio, A. S., Cushing, B. S., & Patisaul, H. B. (2014). A novel model for neuroendocrine toxicology: Neurobehavioral effects of BPA exposure in a prosocial species, the prairie vole (Microtus ochrogaster). *Endocrinology*, en20141379. https://doi.org/10.1210/en.2014-1379

Sun, Y., Huang, H., Sun, Y., Wang, C., Shi, X. L., Hu, H. Y., … Fujie, K. (2013). Ecological risk of estrogenic endocrine disrupting chemicals in sewage plant effluent and reclaimed water. *Environmental Pollution*. https://doi.org/10.1016/j.envpol.2013.05.006

Suzuki, S., & Handa, R. J. (2005). Estrogen receptor-??, but not estrogen receptor-??, is expressed in prolactin neurons of the female rat paraventricular and supraoptic nuclei: Comparison with other neuropeptides. *Journal of Comparative Neurology*, *484*, 28–42. https://doi.org/10.1002/cne.20457

Takagi, Y., Aburada, S., Hashimoto, K., & Kitaura, T. (1986). Transfer and distribution of accumulated (14C) polychlorinated biphenyls from maternal to fetal and suckling rats. *Archives of Environmental Contamination and Toxicology*, *15*(6), 709–715. https://doi.org/10.1007/bf01054917

Takayanagi, Y., Yoshida, M., Bielsky, I. F., Ross, H. E., Kawamata, M., Onaka, T., … Nishimori, K. (2005). Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 16096–16101. https://doi.org/10.1073/pnas.0505312102

Tang, A. C., Nakazawa, M., Romeo, R. D., Reeb, B. C., Sisti, H., & McEwen, B. S. (2005). Effects of long-term estrogen replacement on social investigation and social memory in ovariectomized C57BL/6 mice. *Hormones and Behavior*, *47*(3), 350–357. https://doi.org/10.1016/j.yhbeh.2004.10.010

Thomas, G. M., & Huganir, R. L. (2004). MAPK cascade signalling and synaptic plasticity. *Nature Reviews Neuroscience*, *5*(3), 173–183. https://doi.org/10.1038/nrn1346

Topper, V. Y., Walker, D. M., & Gore, A. C. (2015). Sexually dimorphic effects of gestational endocrine-disrupting chemicals on microRNA expression in the developing rat hypothalamus. *Molecular and Cellular Endocrinology*, *414*, 42–52. https://doi.org/10.1016/j.mce.2015.07.013

Uhlenhaut, N. H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., … Treier, M. (2009). Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell*, *139*(6), 1130–42. https://doi.org/10.1016/j.cell.2009.11.021

Unni, S., Kasiviswanathan, S., D'Souza, S., Khavale, S., Mukherjee, S., Patwardhan, S., & Bhartiya, D. (2012). Efficient cryopreservation of testicular tissue: effect of age, sample state, and concentration of cryoprotectant. *Fertility and Sterility*, *97*(1), 200–8.e1. https://doi.org/10.1016/j.fertnstert.2011.10.018

Veenit, V., Cordero, M. I., Tzanoulinou, S., & Sandi, C. (2013). Increased corticosterone in peripubertal rats leads to long-lasting alterations in social exploration and aggression. *Frontiers in Behavioral Neuroscience*, *7*, 26. https://doi.org/10.3389/fnbeh.2013.00026

Vela, J. (2005). Sexual behavior of female rats in a multiple-partner preference test, *47*, 290–296. https://doi.org/10.1016/j.yhbeh.2004.11.012

Vela, J. (2005). Sexual behavior of female rats in a multiple-partner preference test, *47*, 290–296. https://doi.org/10.1016/j.yhbeh.2004.11.012

Venerosi, A., Ricceri, L., Tait, S., & Calamandrei, G. (2012). Sex dimorphic behaviors as markers of neuroendocrine disruption by environmental chemicals: The case of chlorpyrifos. *NeuroToxicology*, *33*, 1420–1426. https://doi.org/10.1016/j.neuro.2012.08.009

Viau, V., & Meaney, M. J. (1991). Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology*, *129*, 2503–2511. https://doi.org/10.1210/endo-129-5-2503

Walker, D. M., Goetz, B. M., & Gore, A. C. (2013). Dynamic postnatal developmental and sex-specific neuroendocrine effects of prenatal PCBs in rats. *Molecular Endocrinology (Baltimore, Md.)*, 1–17. https://doi.org/10.1210/me.2013-1270

Walker, D. M., Kirson, D., Perez, L. F., & Gore, A. C. (2012). Molecular profiling of postnatal development of the hypothalamus in female and male rats. *Biology of Reproduction*, *87*, 129. https://doi.org/10.1095/biolreprod.112.102798

Walker, D. M., Juenger, T. E., & Gore, A. C. (2009). Developmental profiles of neuroendocrine gene expression in the preoptic area of male rats. *Endocrinology*, *150*, 2308–2316. https://doi.org/10.1210/en.2008-1396

Walker, D. M., Kermath, B. A., Woller, M. J., & Gore, A. C. (2013). Disruption of reproductive aging in female and male rats by gestational exposure to estrogenic endocrine disruptors. *Endocrinology*, *154*, 2129–2143. https://doi.org/10.1210/en.2012-2123

Wang, Z., & De Vries, G. J. (1993). Testosterone effects on paternal behavior and vasopressin immunoreactive projections in priarie voles (Microtus ochrogaster). *Brain Research*, *631*, 156–160. https://doi.org/10.1016/0006-8993(93)91203-5

Weissbrod, A., Shapiro, A., Vasserman, G., Edry, L., Dayan, M., Yitzhaky, A., … Kimchi, T. (2013). Automated long-term tracking and social behavioural phenotyping of animal colonies within a semi-natural environment. *Nature Communications*, *4*, 2018. Retrieved from http://dx.doi.org/10.1038/ncomms3018

Wersinger, S. R., R. Kelliher, K., Zufall, F., Lolait, S. J., O'Carroll, A. M., & Young, W. S. (2004). Social motivation is reduced in vasopressin 1b receptor null mice despite normal performance in an olfactory discrimination task. *Hormones and Behavior*, *46*, 638–645. https://doi.org/10.1016/j.yhbeh.2004.07.004

Williams, S. A., Jasarevic, E., Vandas, G. M., Warzak, D. A., Geary, D. C., Ellersieck, M. R., … Rosenfeld, C. S. (2013). Effects of Developmental Bisphenol A Exposure on Reproductive-Related Behaviors in California Mice (Peromyscus californicus): A Monogamous Animal Model. *PLoS ONE*, *8*. https://doi.org/10.1371/journal.pone.0055698

Williams, S. A., Jasarevic, E., Vandas, G. M., Warzak, D. A., Geary, D. C., Ellersieck, M. R., … Rosenfeld, C. S. (2013). Effects of Developmental Bisphenol A Exposure on Reproductive-Related Behaviors in California Mice (Peromyscus californicus): A Monogamous Animal Model. *PLoS ONE*, *8*. https://doi.org/10.1371/journal.pone.0055698

Witty, C. F., Foster, T. C., Semple-Rowland, S. L., & Daniel, J. M. (2012). Increasing Hippocampal Estrogen Receptor Alpha Levels via Viral Vectors Increases MAP Kinase Activation and Enhances Memory in Aging Rats in the Absence of Ovarian Estrogens. *PLoS ONE*, *7*(12), 1–10. https://doi.org/10.1371/journal.pone.0051385

Wolf, C., Gerald Leblanc, Ostby, J., & Gray, L. (2000). Characterization of the Period of Sensitivity of Fetal Male Sexual Development to Vinclozolin. *Toxicol. Sci.*, *55*(1), 152–161.

Wolstenholme, J. T., Goldsby, J. A., & Rissman, E. F. (2013). Transgenerational effects of prenatal bisphenol A on social recognition. *Hormones and Behavior*, *64*, 833–839. https://doi.org/10.1016/j.yhbeh.2013.09.007

Wolstenholme, J. T., Taylor, J. A., Shetty, S. R. J., Edwards, M., Connelly, J. J., & Rissman, E. F. (2011). Gestational exposure to low dose bisphenol a alters social behavior in juvenile mice. *PLoS ONE*, *6*. https://doi.org/10.1371/journal.pone.0025448

Young, W. S., Li, J., Wersinger, S. R., & Palkovits, M. (2006). The vasopressin 1b receptor is prominent in the hippocampal area CA2 where it is unaffected by restraint stress or adrenalectomy. *Neuroscience*, *143*(4), 1031–1039. https://doi.org/10.1016/j.neuroscience.2006.08.040

Zhang, W., Sargis, R. M., Volden, P. A., Carmean, C. M., Sun, X. J., & Brady, M. J. (2012). PCB 126 and other dioxin-like PCBs specifically suppress hepatic PEPCK expression via the aryl hydrocarbon receptor. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0037103

Zingg, H. H., & Lefebvre, D. L. (1988). Oxytocin and vasopressin gene expression during gestation and lactation. *Brain Res*, *464*